Functional Interactions and Evolution of cAMP-PKA Signaling in *Saccharomyces*

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
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ABSTRACT

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Abstract

In an attempt to gain more insight on functional evolution of cAMP-PKA pathway I have taken a comparative approach and examined functional interactions of cAMP-PKA signaling in well-studied yeast developmental programs and closely related *Saccharomyces sensu stricto* species. I have shown that variation in cAMP-PKA signaling contributes significantly to variation in developmental responses in *Saccharomyces cerevisiae*. Variation in pseudohyphal growth and sporulation, two inversely correlated developmental strategies to nutrient limitation in yeast, proportional to variation in intracellular cAMP levels. *S. cerevisiae* strains proficient in pseudohyphal growth have higher intracellular cAMP concentrations relative to strains that sporulate efficiently. Phenotypic, genetic and signaling data presented here suggest that the cAMP-PKA signaling underlies a phenotypic trade-off between sporulation and pseudohyphal growth in *S. cerevisiae.*

Further investigation into the role of cAMP-PKA signaling in closely related *Saccharomyces paradoxus* and *Saccharomyces bayanus* revealed an antagonistic function of cAMP-PKA signaling for developmental responses in *S. bayanus.* Unlike in *S. cerevisiae*, increased cAMP concentrations surprisingly inhibit pseudohyphal response in *S. bayanus*. Another unanticipated finding in this work is that in *S. bayanus*, Flo11, required for pseudohyphal differentiation in *S. cerevisiae,* is dispensable. Additionally, interactions of cAMP-PKA signaling and the general-stress response mechanism appear

reversed in *S. bayanus*. As shown by deletion mutation, gene expression and pharmacological treatment data, altered interactions and alternative targets downstream of cAMP-PKA could critically contribute to alternative regulation of nutrient-induced development in *S. bayanus.*

Intracellular cAMP concentrations show decaying oscillations upon glucose replenishment in derepressed yeast cells. The quantitative characteristics of oscillations are distinct within and between *Saccharomyces* species. Given the tight regulation of cAMP levels and its critical role, the variation in cAMP oscillatory dynamics could be reflective of differential interactions of cAMP-PKA signaling that also underlie induction of developmental programs to changing environments. As such, intracellular cAMP levels and dynamics could potentially be used as molecular phenotypes.

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Dedication

I dedicate this thesis to my cherished grandparents, Cemile and Zeki Ural.

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1. Introduction

1.1 The cyclic AMP- PKA signaling

The mechanism of cyclic adenosine monophosphate (cAMP)–Protein Kinase A (PKA) is one of the well-studied signal transduction pathways. The cAMP-PKA signaling has provided a fundamental paradigm for our understanding on how external signals are transmitted to the cellular machinery to elicit physiological responses. Major breakthroughs elucidating the components and action mechanism of cAMP-PKA pathway have formulated the concept of second messenger signaling. With the discovery of the second messenger cAMP (1957) the research into how an external signal triggers a cellular response led to the finding of PKA and reversible phosphorylation (1968), G-proteins (1971), and G-protein coupled receptors (1980) that together established an outline for signal transduction mechanisms[1–4].

Extensive and expanding studies have shown that cAMP-PKA signaling plays a pivotal role in various biological functions in a range of cell types and organisms. Its actions critically regulate metabolism, cell growth and differentiation, and gene expression that underlie cellular and organismal processes including hormone actions, oogenesis, muscle relaxation, and neurodevelopment [5]. Well-characterized differential actions of the cAMP-PKA pathway in response to diverse stimuli mediating opposing and/or complementary responses in unicellular and multicellular fungi and animals underscore the versatility and importance of this signaling pathway. However, despite the wealth of information on cAMP-PKA signaling mechanism that has helped us understand health and disease states, our understanding of its functional interactions

and evolution is incomplete. Examining functional evolution of such a well-studied signaling mechanism could further insights on how integration and coordination of signals by regulatory networks govern decision-making processes and physiological responses of a cell. Understanding evolution of functional interactions regulating response-decisions is a central theme of this thesis.

1.1.1 Basic structure and mechanism of the cAMP-PKA signaling

Basic structure and mechanism of cAMP-PKA signaling is conserved from yeast to man[5]. Canonically, the cAMP-PKA signaling is triggered when an extracellular ligand engages a GPCR that subsequently activates its associated heterotrimeric G-protein. The activated G-protein stimulates the membrane-bound adenylate cyclase (AC), which catalyzes synthesis of cAMP from ATP. PKA is a tetrameric holoenzyme consisting of two catalytic subunits bound a regulatory subunit dimer. An increase in cAMP levels results in binding of cAMP molecules to each regulatory subunit at a 2:1 ratio, and dissociating and activating the catalytic subunits to phosphorylate a variety of target proteins, eventually modifying biological activity [6]**.** The intracellular levels of cAMP are regulated by the balance between synthesis and hydrolysis activity of AC and phosphodiesterases (PDEs), respectively [7,8]

While growth factors, such as hormones and neurotransmitters, act as initiators of the cAMP-PKA signaling in multi-cellular organisms, environmental nutrients can also directly trigger the pathway in unicellular organisms for rapid induction of developmental responses. For example, in the yeast *Saccharomyces cerevisiae*, glucose binds to a nutrient sensing GPCR (Gpr1) and activates AC (Cyr1) through its cognate G-

protein (Gpa2). Another G-protein system that also activates AC in yeast includes Ras proteins that have been suggested to be important for intracellular sensing of glucose. Inactive PKA in *S. cerevisiae* is also a tetramer composed of a homodimer regulatory subunit (Bcy1) and two of the catalytic subunits (Tpk1, Tpk2, and Tpk3) [9–12]**.** Activation of PKA involves cAMP binding to Bcy1 which in turn causes a conformational change that releases the catalytic subunits that can function synergistically or antagonistically in numerous overlapping and distinct cellular processes (Figure1) [13,14].

1.1.2 Role and Interactions of the cAMP-PKA Pathway in Nutrient Signaling

How cells perceive environmental nutritional conditions is a critical determinant of cellular growth, development, and differentiation. The cAMP-PKA signaling plays a central role in sensing nutrient levels and significantly regulates subsequent modulation of transcriptional, metabolic, and developmental programs under starvation and nutrient-rich conditions. The cAMP-PKA signaling promotes growth and development primarily in response to carbon sources. Glucose positively stimulates the activity of cAMP-PKA pathway that drives growth rates, mass accumulation, cell cycle progression and proliferation [15–19].

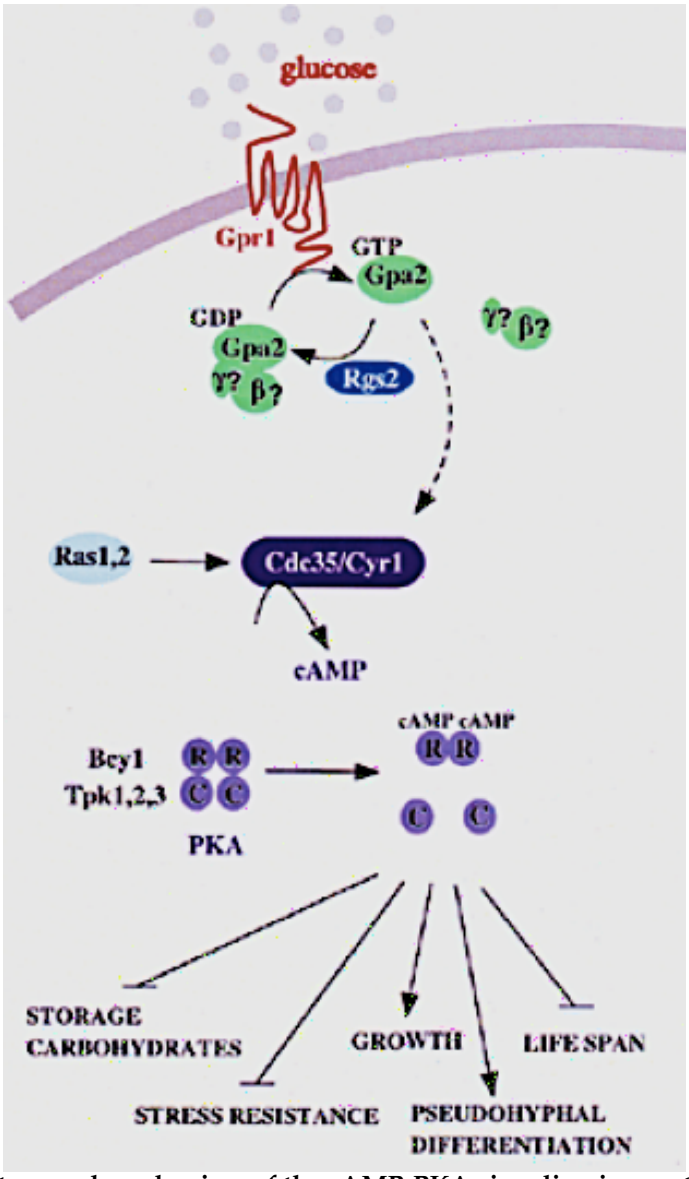

Figure 1 Basic structure and mechanism of the cAMP-PKA signaling in yeast

The cAMP-PKA signaling transmits the encoded nutrient signal to the cellular machinery together with also nutrient-regulated Target of Rapamycin (TOR) and SNF1/AMPK pathways (Figure 2). These evolutionarily conserved signaling mechanisms collectively orchestrate cellular responses to fluctuating quality and quantity of nutrient to ensure cell survival from yeast to man (REF). The coordinative activities of cAMP-PKA and nitrogen-regulated TOR signaling are predominant drivers

of cell growth and proliferation when nutrient levels are optimal. Moreover, under these conditions, parallel functions of the two pathways also target a similar set of substrates and represses stress-induced responses [20–23]. On the other hand, under low glucose levels or in the presence of alternative carbon sources the cAMP-PKA pathway derepresses and activates SNF1/AMPK pathway, central regulator of energy metabolism [24–27]. Additionally, upon nutrient-limitation the cAMP-PKA signaling derepresses stress-induced transcription factors and triggers appropriate stress responses [28–32].

Dysregulation of cAMP-PKA signaling and consequent mismatch in cells' perception of nutritional states impinges on growth rates and developmental responses. As suchThe cAMP-PKA signaling transmits the encoded nutrient signal to the cellular machinery together with also nutrient-regulated Target of Rapamycin (TOR) and SNF1/AMPK pathways (Figure 2). These evolutionarily conserved signaling mechanisms collectively orchestrate cellular responses to fluctuating quality and quantity of nutrient to ensure cell survival from yeast to man [33,34].

1.2 Nutrient-induced Developmental Responses in Yeast

Yeast cells respond to changing nutrient conditions by inducing key developmental responses. In order to survive unpredictable nutrient availability in nature yeast cells undergo filamentous differentiation, form complex structures at colony level or sporulate [35]. These responses are primarily activated under carbon and/or nitrogen deficiency. In this work I focus on pseudohyphal growth, a form of filamentous response, and sporulation.

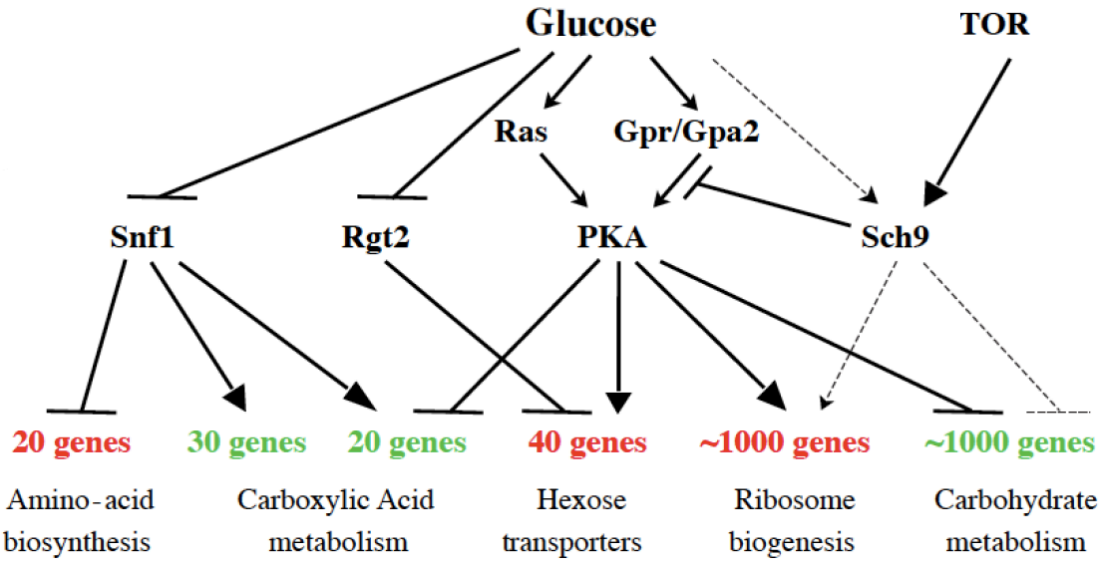

Figure 2 Diagram of a nutrient responsive network in yeast
Regulatory interactions are shown as reported in *S. cerevisiae*. Dotted lines indicate indirect connections[25].

1.2.1 Pseudohyphal growth

Diploid *S. cerevisiae* cells undergo pseudohyphal differentiation in response to nitrogen limitation when a fermentable carbon source, such as glucose, is present (Figure 3a). Under these conditions cells elongate, physically remain connected and acquire the ability to adhere and invade substrates they occur on. The carbon signaling underlying the response involves the cAMP-PKA and MAPK pathways, as well as Snf1 and TOR signaling Glucose triggers the cAMP-PKA and MAPK signaling via Ras2 protein and activated modules of the pathways exert their kinase activities on various proteins. The global regulator of carbon metabolism, Snf1, and elements of nitrogen metabolism in TOR1/Sch9 pathway process the carbon signal to induce pseudohyphal response by interacting with PKA [35–38].

Under limiting nitrogen and ample glucose conditions, elevated intracellular cAMP levels activate PKA catalytic subunits, Tpk1, 2 and 3. The subunits are redundant

for viability, however, they play distinct roles in pseudohyphal response [13,14]. It has been shown that Tpk2 positively regulates the response by directly suppressing the transcriptional repressor Sfl1 and directly activating the transcriptional activator Flo8 to induce expression of Flo11 [39]. In contrast to Tpk2 mutants, which show a diminished pseudohyphal response, mutations in Tpk3 have been observed to enhance pseudohyphal growth. Similarly, the Tpk1 subunit has been reported to have inhibitory effect on the pseudohyphal response [13,40]**.** This cascade of interactions leads to differential regulation of many genes, perhaps the best studied being *FLO11*, a gene that encodes a cell surface glycoprotein required for adhesiveness/ invasiveness and which is essential for pseudohyphal growth [41–43]. Because the key steps in this developmental program have been well established it represents a powerful platform to study the functional evolution of molecular signaling events.

Pseudohyphal growth is related to dimorphic switch between yeast and hyphal forms observed in many fungal species. Research on *S. cerevisiae* pseudohyphal growth of has contributed significantly to progress in identification of signaling pathways regulating filamentous differentiation observed in medically, agriculturally, and biotechnologically important fungi, such as *Candida albicans*, *Cryptococcus neoformans*, *Ustilago maydis*, and *Yarrowia lypolytica* [44–50]. The cAMP –PKA and MAPK signaling pathways are critical regulators of filamentous growth in fungi despite their adaptation to drastically different niches. For example, in *S. cerevisiae* (adapted to tree barks and bruised fruit surfaces) and in *C. albicans* and *C. neoformans* (animal pathogens adapted to host conditions) signaling components and interactions of cAMP-PKA and MAPK

signaling promote filamentous differentiation [37,47,51–53]. MAPK signaling positively regulates filamentous transition also in *U. maydis* (a plant pathogen) and *Y. lypolytica* (occurs in oil-rich surfaces) whereas cAMP-PKA pathway inhibits the transition in response to nutrient limitation in these species[54,55]. The opposing effects of cAMP-PKA signaling on a critical developmental response underscore the pivotal position of cAMP-PKA in nutrient-induced developmental networks and how altered interactions between conserved components can lead to different outcomes.

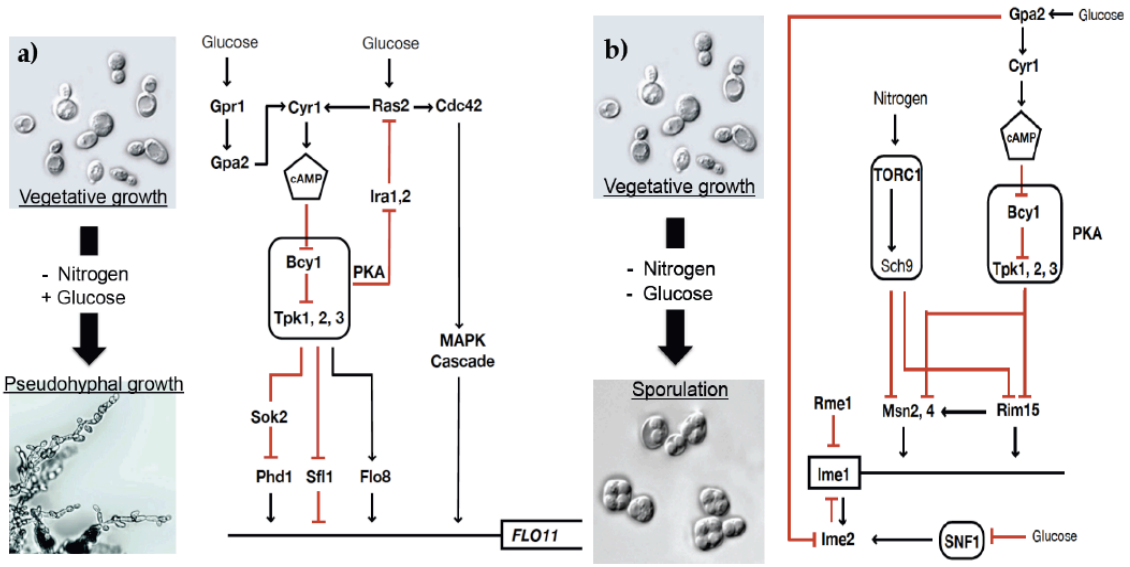

Figure 3 Developmental responses to nutrient limitation in yeast
a) A summary of interactions regulating pseudohyphal growth **b)** A summary of interactions regulation sporulation (Red bars: Inhibition, Black arrows: Activation

1.2.2 Sporulation

Sporulation is an alternative response in diploid yeast cells to limiting nitrogen levels. This developmental response involves meiosis (generation of haploid cells) followed by formation of stress-resistant gametes called spores. In addition to nitrogen depletion, sporulation requires the absence of glucose and the availability of a non-fermentable carbon source, such as acetate (Figure 3b). These nutritional signals initiate

meiosis when integrated on promoters of IME1, a transcription factor, and IME2, a serine-threonin kinase. The actions of Ime1 and Ime2, together with the nutrient signals, maintain progression of sporulation by tightly controlled sequential processes. Post-meiotic reactions during sporulation proceed upon combinatorial and alternative interactions of these signals [35,56–58].

Control of sporulation by carbon signaling involves the cAMP-PKA, Snf1 and RIM101 pathways[35,59]. cAMP-PKA signaling contributes to glucose inhibition of meiosis by activating repressors and inhibiting activators such as, Msn2/4 and Rim15. In the presence of glucose, deactivated Snf1 blocks meiotic initiation as a central component of glucose repression pathway. On the other hand, when activated in presence of non-fermentable carbon sources Snf1 promotes full induction of IME1. A non-fermentable carbon source promotes meiosis also by stimulating respiratory metabolism. Respiration increases extracellular alkalinization, which is sensed and mediated by RIM101 signaling to IME1. Localization and stability of Ime1 is influenced by nitrogen-sensing TOR pathway that also promotes meiosis by upregulation of G1 cyclins [60].

1.3 Overview of Saccharomyces sensu stricto

1.3.1 Phylogeny *of Saccharomyces* species

The *Saccharomyces sensu stricto* lineage is a useful resource to obtain a detailed view of dynamic communications between signaling cascades. Evolutionary distances between the species are close enough to zoom in on details of interactions that determine developmental responses in a signal- and species-specific manner. Species in the group

share a core of functionally conserved regulatory mechanisms with dynamic connections evolved for rapid adaptation to changing environmental conditions [61–63]. These features make the *Saccharomyces sensu stricto* ideal to study interactions between signaling pathways and their evolution.

The *Saccharomyces sensu stricto* are ascomycete yeasts (subphylum *Saccharomycotina*, phylum Ascomycota), and the monophyletic *Saccharomyces genus* currently includes seven species: *S. cerevisiae, S.paradoxus, S.mikatae, S.kudriavzevii, S. arboricolus, S. uvarum* and *S. eubayanus* (Figure 4) [63–65]. The phylogenetic relationships between members of the *Saccharomyces* genus have been controversial since the group was first proposed [66]. The species in the genus had been reclassified and reassigned successively based on biochemical and molecular identification techniques until construction of multi-locus and genomic phylogenies and recent identification of *S. eubayanus,* a parental species of hybrids in *S. bayanus* species complex. The *S. bayanus* species complex includes the proper *S. uvarum* and *S. eubayanus* as well as naturally occurring double and triple hybrids of *S. cerevisiae, S. uvarum* and *S. eubayanus)*[63,67].

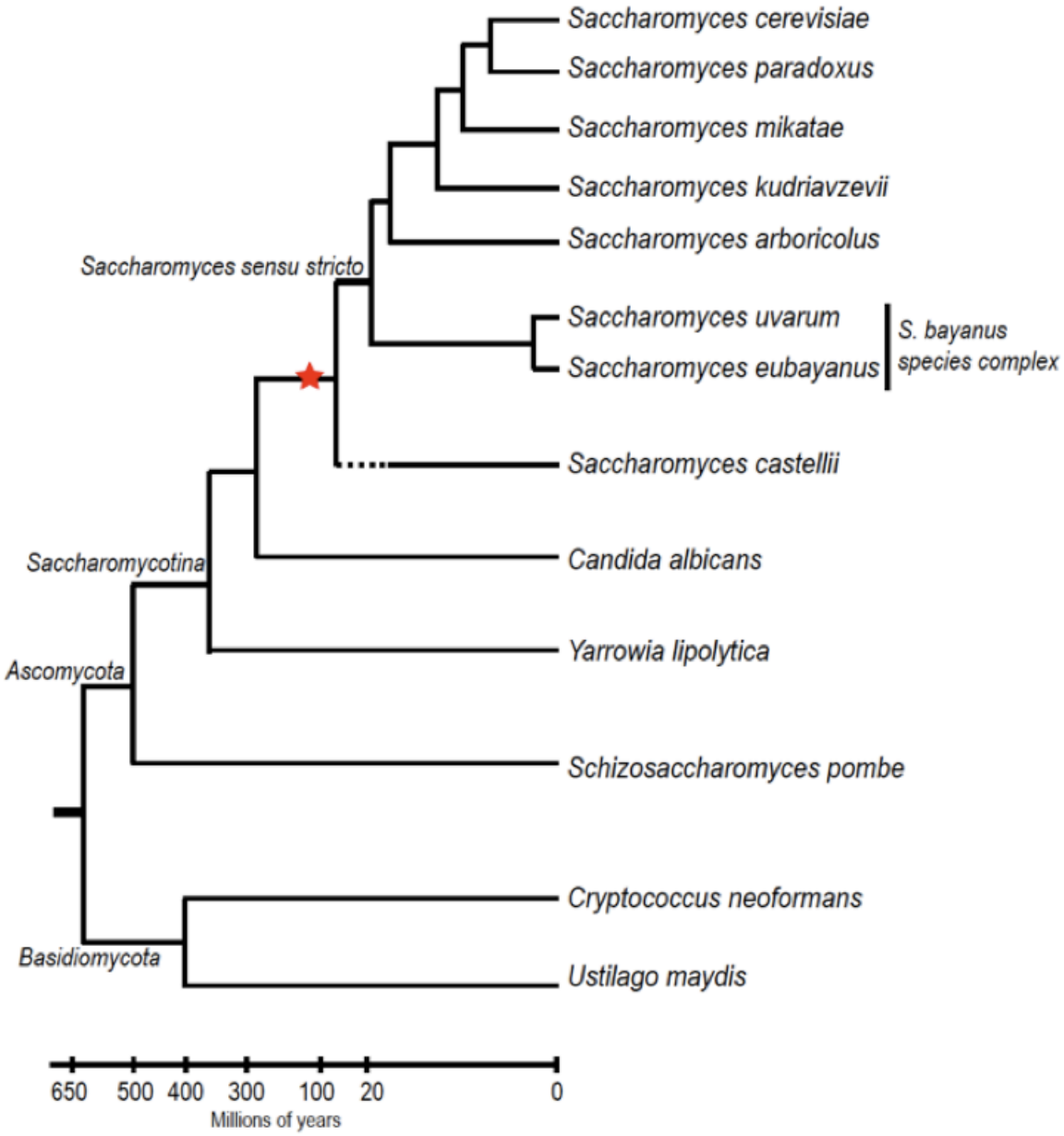

Figure 4Overview of phylogenetic relationships in fungi
The figure summarizes approximate phylogenetic relationships between species belong to two major fungal subphyla. The chronogram shows estimated divergence times between the lineages. The star (★) indicates the likely position of the whole-genome duplication. The branch lengths are arbitrary

The famous lager yeast S. *pastorianus* (*S. carlsbergensis*), a domesticated hybrid species between *S. cerevisiae* and *S. eubayanus*, is also belongs to the *S. bayanus* complex [68,69].

The nomenclature of *S. bayanus* and *S. uvarum* has been confusing and controversial for decades, and since identification of *S. eubayanus*, *"S. uvarum"* has been proposed to be used as descriptor of a distinct species while "*S. bayanus"* to indicate the *S. bayanus* species complex. In this work I opt to use *"S. bayanus"*. Yet, I emphasize that the strain background studied in Chapter 3 has been shown to be *S. uvarum* by and this has been also confirmed by PCR identification during the work [70,71]

The species are estimated to have diverged 5-20 million years ago (MYA) from an ancestor that underwent whole genome duplication (WGD) about 100 MYA [72,73]. This WGD event is hypothesized to be the basis of their remodeled metabolism that has brought about unique traits, such as fermentation abilities in presence of oxygen, which characterizes their life styles [64,74]**.** Beside whole genome duplication, *Saccharomyces sensu stricto* share several common genomic features. For example, their genomes show highly conserved synteny and have approximately 6000 genes that are positioned on 16 chromosomes [75–77]**.**

1.3.2 Ecology of *Saccharomyces*

Information on the habitats and population genetics of *Saccharomyces sensu stricto* species is limited. Until recently, as reflected in the genus name "*Saccharomyces"*, the species were thought to be mostly found in sugar-rich and human-associated environments However, recent studies have demonstrated that members of the *Saccharomyces* lineage inhabit both human-associated and uncultivated natural

environments that are not necessarily rich in sugar [63,66]. Furthermore, *Saccharomyces* species are found in sympatry; presumably facilitated by their preferences for different growth temperatures [78,79]. Presence of the *Saccharomyces* in diverse environments, such as tree barks and fluxes, decaying plants and soil are reflective of their distinct physiologies and response mechanisms evolved to adapt to their diverse habitats.

2. The cAMP-PKA pathway underpins a life-history trade-off in *Saccharomyces cerevisiae*

2.1 Introduction

Signaling pathways that are conserved, shared across taxa, and responsible for coordinating numerous cellular responses are critical for mediating life-history of an organism [35,80,81]. A central topic in life-history studies is life-history trade-offs, which have been shown in many ways in a range of organisms. Environmental and physiological cues influence life-history traits and trade-offs extensively. Such cues are sensed, processed and integrated by molecular signaling mechanisms in cells to coordinate growth, metabolism, and stress responses for maximal fitness [82]. Life-history models traditionally explain trade-offs by resource and energy allocation; however, there are a number of studies that also show that trade-offs can be mediated by molecular signaling pathways. For example, in *Caenorhabditis elegans* and *Drosophila melanogaster*, mutations in nutrient responsive Insulin/Insulin-like signaling (IIS) have been shown to modulate a trade-off between survival and reproduction, a classic example of life-history trade-off **[83,84].** Similarly, the nutrient signaling pathway TOR signaling has been shown to mediate longevity via dietary restriction in various organisms [85,86]. Such studies have provided insights on quantitative and mechanistic underpinnings that determine and/or modulate trade-offs. To further understanding in mechanistic basis of life-history trade-offs a number of model organisms for which we have a good grasp of molecular signaling seems to offer a good platform.

The yeast, *Saccharomyces cerevisiae*, has played a pivotal role in our understanding of major cellular and molecular processes of the eukaryotic cell. This unicellular, diploid eukaryote occurs in a wide range of niches, and has been isolated from both cultivated and uncultivated environments. Wild isolates have been collected from oak tree microhabitats, such as soil and bark, as well as damaged/ decaying fruits and beetle guts [87–89]**.** By the same token, domesticated populations of the yeast have long been associated with various human activities including baking, brewing, and wine making. In addition, emergence of *S. cerevisiae* as an opportunistic pathogen in clinical studies highlights the breadth of ecological niches this yeast species inhabits[90].

Yeast cells, unlike in the lab, encounter harsh conditions in their natural habitats in which quantity and quality of nutrients often fluctuate and are suboptimal. In yeast, pseudohyphal growth and sporulation represent two life-history strategies for survival under nutrient stress. Nitrogen depletion when glucose is abundant promotes transition to pseudohyphal growth in which cells continue to grow vegetatively as they become more adherent and invasive; this form of growth reflects the search for nutrients. Extreme deficiency in both nitrogen and glucose, on the other hand, induces sporulation during which cells exit vegetative growth, and undergo meiosis, generating haploid spores. Magwene et.al. (2010) has shown an inverse correlation between these developmental strategies, and reported a trade-off between pseudohyphal growth and sporulation in yeast [91].

Recently, there have been a number of studies that investigated life-history trade-offs in this well-studied model eukaryote at molecular level. Spor et. al. **(2008)** has

reported that glucose consumption rate is a determinant of a trade-off between growth and carrying capacity [92]. In this study, Spor et. al. proposed "ant" and "grasshopper" strategies for populations with low growth rate and high carrying capacity, and with high growth rate and low carrying capacity, respectively. The same group also has showed that glucose metabolism underlies the two life-history strategies, and proposed that antagonistic pleiotropy between glycoliytic genes drives the switch between the ant and grasshopper strategies[93]. Similarly, earlier work by Magwene et.al. (2010) lab highlighted a trade off between sporulation and pseudohyphal response that is reflected in expression of a transcription factor RME1, which echoes the premise that the negative correlation between components of fitness could due to be loci that are in antagonistic pleiotropy [91]. Altogether, these studies point to molecular mechanisms at metabolic and gene expression levels as proximal causes of life-history trade-offs. However, although implicated in several studies, so far there are no records of a work that has looked at signaling pathways to investigate molecular basis of trade-offs in yeast

The cyclic AMP-PKA signaling is a major pathway that regulates growth, development, metabolism and stress resistance in yeast. This evolutionarily conserved signaling pathway exerts its effects via activation and inhibition of transcriptional elements and molecular switches. In *S. cerevisiae*, cAMP-PKA signaling is essential for pseudohyphal growth and its critical role for pseudohyphal response is well characterized [13,36,39,94]. At the same time active cAMP-PKA inhibits sporulation (Figure 5) [9,95].

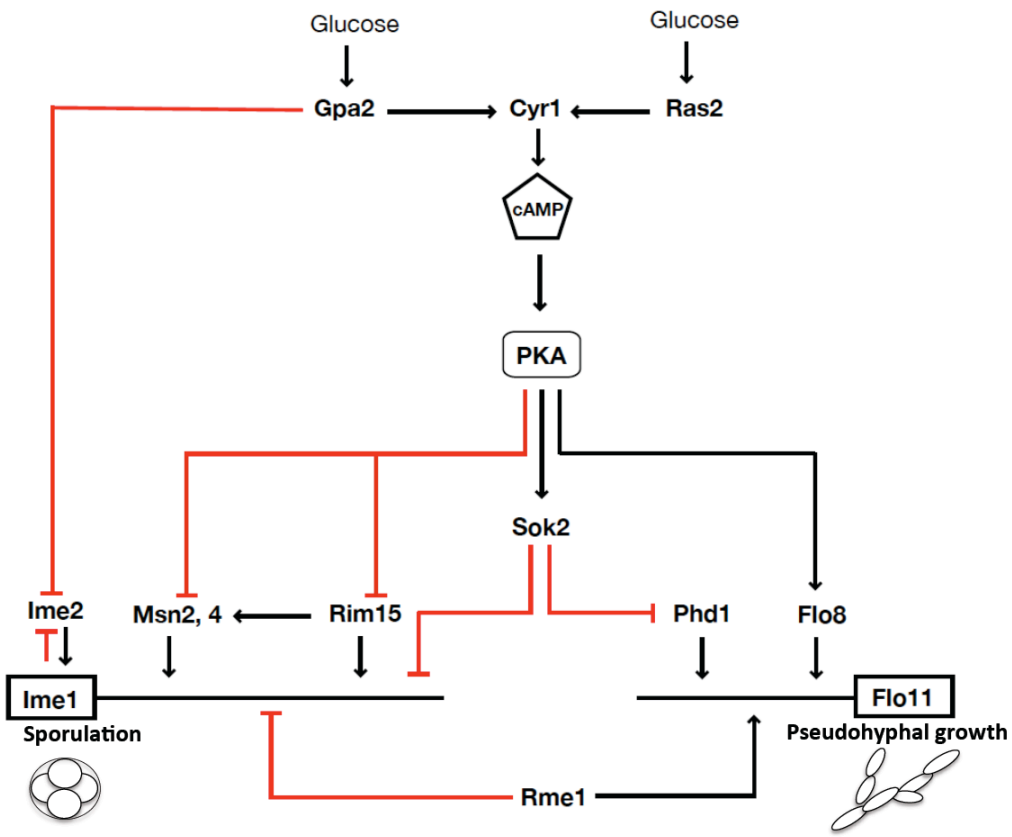

Figure 5 Pleiotropic cAMP-PKA interactions in yeast developmental responses
The cAMP-PKA pathway promotes growth and development, such pseudohyphal response, whereas inhibits stress induced responses, such sporulation

The cAMP-PKA signaling pathway has been proposed as a possible underlying mechanism for life history trade-offs observed in *S. cerevisiae*. Spor et. al**.(2008)** suggested that while cAMP-PKA pathway triggers activation of glycolytic enzymes, it inhibits cell-cyle progression, leading to a small carrying capacity in yeast populations[92]. Likewise, mutations in elements of cAMP-PKA pathway have been identified as possible modulators of a trade-off under optimal carbon source conditions under limited nitrogen [96]. These studies clearly point to the central role of cAMP-PKA signaling for survival in diverse environments by regulating growth and reproduction.

In this work I integrate knowledge of well-studied cAMP-PKA pathway on a life-history trade-off in *S. cerevisiae*, and show that variation in this pathway mirrors variation observed in nutrient induced developmental responses in yeast. I propose that the cAMP-PKA pathway is a critical regulator of a life-history trade-off between pseudohyphal growth and sporulation in *S. cerevisiae.*

2.2 Materials and Methods

Strains, media and phenotyping

Strains used are given in (Table 1). Yeast strains were grown overnight in YPD medium to a density of 2 x 10^7 cells/mL. The cells were washed twice in sterile water and transferred to agar plates (10^6 cells were plated for both sporulation and pseudohyphal growth). The sporulation media (SM) used is composed of 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, and 2% Bacto agar. Pseudohyphal growth was assayed using a modified SLAD medium [97] consisting of 0.17% YNB − AA/AS, 1% dextrose, 50 μM ammonium sulfate, and 2% Noble agar. Strains were incubated at 30°C and scored 72 h after transfer to the appropriate medium. Sporulation was quantified as the percentage of sporulated cells, and pseudohyphal growth was scored as a binary trait.

Intracellular cAMP assay

Intracellular cAMP concentrations were determined for strains grown in liquid SLAD media for 4 hr at 30°C. 34 μL of cell culture was fixed in 300 μL 1 M n-butanol saturated formic acid, and lysed by four freeze-thaw cycles in dry ice. The formic acid was then evaporated using a Speed Vac Concentrator (Savant Instruments, Hickville)

Table 1 Surveyed *S. cerevisiae* strains (Chapter 2)

The isolates are originated from different niches representing a wide range of environments *S. cerevisiae* occurs.

Strain Number	Strain Name	Origin	Strain Number	Strain Name	Origin
PMY 011	YPS 602	Oak	PMY 123	SK1	Laboratory
PMY 012	YPS 606	Oak	PMY 127	YJM 128	Clinical
PMY 014	YPS 623	Oak	PMY 128	YJM 145	Clinical
PMY 015	YPS 630	Oak	PMY 129	CBS 1227	Clinical
PMY 017	YPS 670	Oak	PMY 131	YJM 222	Clinical
PMY 018	YPS 681	Oak	PMY 133	YJM 224	Distillery
PMY 070	EM93	Fig	PMY 137	VMC 132B	Clinical
PMY 072	PMY 072	Vineyard	PMY 140	YJM 277	Clinical
PMY 074	PMY 074	Vineyard	PMY 141	90-59	Clinical
PMY 083	PMY 083	Vineyard	PMY 144	YJM 311	Clinical
PMY 084	PMY 084	Vineyard	PMY 147	YJM 334	Vineyard
PMY 086	PMY 086	Vineyard	PMY 13	YPS 615	Oak
PMY 087	PMY 087	Vineyard	PMY 118	YJM 440	Clinical
PMY 088	PMY 088	Vineyard	PMY 125	RM11	Vineyard
PMY 093	PMY 093	Vineyard	PMY 130	CBS 1464	Clinical
PMY 094	PMY 094	Vineyard	PMY 132	YJM 223	Clinical
PMY 095	PMY 095	Vineyard	PMY 142	YJM 309	Clinical
PMY 110	PMY 110	Vineyard	PMY 327	YJM 978	Clinical
PMY 111	PMY 111	Vineyard	PMY 328	DBVPG1788	Soil
PMY 112	PMY 112	Vineyard	PMY 330	UWOP3-461.4	Palm nectar
PMY 113	YJM 336	Clinical	PMY 332	L-1374	Wine
PMY 116	YJM 431	Laboratory	PMY 336	YPS128	Oak
PMY 119	YJM 454	Clinical	PMY 338	Y12	Wine
PMY 120	F4852	Clinical	PMY 339	L-1528	Wine
PMY 121	Y55	Laboratory			

NY). cAMP levels were quantified following the Non-Acetylation EIA protocol for the cAMP Biotrak EIA kit (RPN225, GE Healthcare).

RNA isolation and gene expression measurements

Total RNA was isolated from cells grown in SLAD media using the hot acid phenol protocol[98]. cDNA was generated using an iScript cDNA synthesis kit (BioRad). Gene expression was measured by quantitative RT-PCR using the iQ SYBR Green kit (BioRad). qPCR reactions were carried out on a Rotor-Gene 6000 real-time cycler (Corbett Research). I used the ACT1 gene as a "housekeeping" gene to normalize RME1 expression values. For the strains and growth conditions ACT1 exhibits relatively small inter-strain variation and thus is a good basis for normalization. Primers for the qPCR reactions are shown in (Table 2). Primers were synthesized by Sigma-Aldrich. Relative expression of RME1 was calculated as $\log_2 (E_R^{Ct} - E_T^{Ct})$, where E_R and E_T are the amplification efficiencies of the reference (ACT1) and the target gene (RME1); and Ct is the measured cycle thresholds for each gene during the qPCR reaction[99] .

Sanger sequencing

Primers for RAS2 and RME1 were designed in regions lacking polymorphic sites. Primers used are given in (Table 2). The two loci and their promoter regions were sequenced for all 36 strains at the DNA Sequencing Facility at Duke University on an Applied Biosystems 3730xl DNA Analyzer. Sequences were assembled and edited using CodonCode Aligner v2.0.

Table 2 List of primers for qPCR and sequencing reactions

Primer name	Primer Sequence	Purpose
ACT1_F	CCTTCTGTTTTGGGTTTGGA	qPCR
ACT1_R	AGCGGTGATTTCCTTTTGC	qPCR
CYR1_215_F	TTCCCTAACGCAATGAGGAC	qPCR
CYR1_215_R	CAGCCGTCTTGAACTGAGG	qPCR
FLO8_180_F	TTGCTGATGAAACCTCTGC	qPCR
FLO8_180_R	CGGTCCTTGGTCTTCAACC	qPCR
FLO11_103_F	ACAACCAAGTCCATCCCAAC	qPCR
FLO11_103_R	TTGAAATAGGGTTGGTTGCTG	qPCR
GPA2_107_F	ACAGCGTTCCGAAAGAAAA	qPCR
GPA2_107_R	CTTGTCCTCCATCAGCGTC	qPCR
IME1_118_144_186_F	ACCCATTAGAAATTGCCTTCG	qPCR
IME1_144_R	CATTAGAACTGCTGAAGGAGTAAGC	qPCR
IME2_205_F	AGCAGACAACATGGAACAGC	qPCR
IME2_205_R	AAACGAATCGCCTAAAGAGG	qPCR
PHD1_156_F	CCAAGTAGAGGCGAACGGTA	qPCR
PHD1_156_R	ATGGAGCCAATCTTCACGAC	qPCR
RAS2_81_F	AGCACAACTGTCGTGAATGC	qPCR
RAS2_81_R	CTTACCATTTGTCGCCTGG	qPCR
RME1_152_F	TCCAACAAGCAACTGGTCTG	qPCR
RME1_152_R	TTTGCGCCTAAACGTTTTC	qPCR
SOK2_91_F	CCGAACGAACCTAGTGAAGC	qPCR
SOK2_91_R	TTTCTTCGTTGGAGACATCG	qPCR
TPK1_193_F	TCGTTGGTATGCTCCACC	qPCR
TPK1_193_R	AGTTTATTGCCCAAGCGAGA	qPCR
TPK2_144_F	CATCAGGTATCGGTGACACG	qPCR
TPK2_144_R	ATCATCGCCTTGAATACC	qPCR
TPK3_184_F	CCTCCCATTGTGATTGGAAC	qPCR
TPK3_184_R	TGGAAGAAACAGCGGAAAG	qPCR
RAS2_pro2_F	CTCCTAGTTGATTAATATAGTGC	Sequencing
RAS2_pro2_R	GGCAAGTGGTATTGCGACG	Sequencing
RME1_pro2_F	GGCTGAAGGGTATGATTTC	Sequencing
RME1_pro2_R	GCTGGAAGGCAGGCTGG	Sequencing

2. 3 Results

Pseudohyphal growth and sporulation inversely correlated

Phenotypic assays conducted on 49 isolates of different origins exhibited a trade-off such that strains with greater than 40% sporulation efficiency displayed poor pseudohyphal growth or none. Conversely, strains with various levels of pseudohyphal response (scored as 1) showed poor sporulation efficiency (Figure 6). Of these 36 strains, 20 had pseudohyphal response with low sporulation efficiency (<40%), and 12 were non-psh with high sporulation efficiency (>40%). In addition, there were four non-responsive strains, three of which had inefficient pseudohyphal and sporulation responses, and the remaining one strain was proficient in both.

Intracellular cAMP levels correlate with pseudohyphal response propensity

To investigate whether cAMP levels play a critical role in the trade-off between the two developmental responses I measured intracellular cAMP levels for the panel strains under nitrogen limiting conditions (SLAD-1%, 4 hours). The levels of cAMP ranged between 15-142 fmol/106 cells in which cAMP concentrations were higher in pseudohyphal strains than in sporulation-efficient strains ($p < 0.01$) (Figure 7a). To test whether cAMP measurements are predictive of pseudohyphal response and sporulation efficiency I regressed both phenotypes on cAMP concentrations using a logistic regression model ($p < 0.01$). As shown in (Figure 7b) the model predicts that strains with cAMP concentrations higher than 50 fmol/106 cells have greater than 40% probability of being pseudohyphal, while on the other hand strains with less than 50 fmol/106 cells of intracellular cAMP levels are most likely to be strong sporulators.

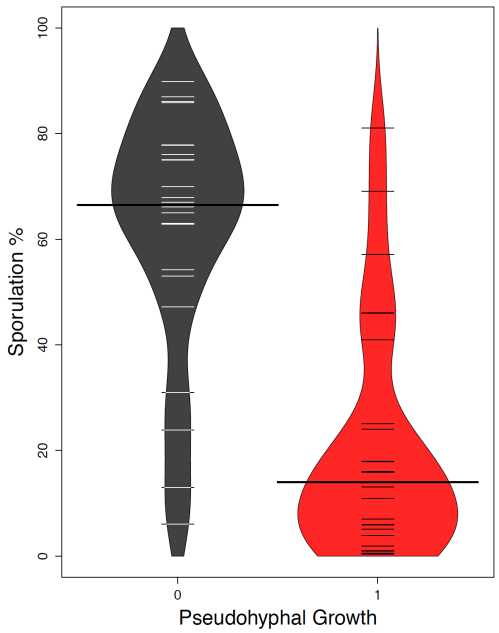

Figure 6 A Life-history trade-off in *S. cerevisiae*
Joint bean plots of sporulation as a function of pseudohyphal growth show inverse correlation between the developmental responses. Tick marks represent strains and the median is marked with a black horizontal bar

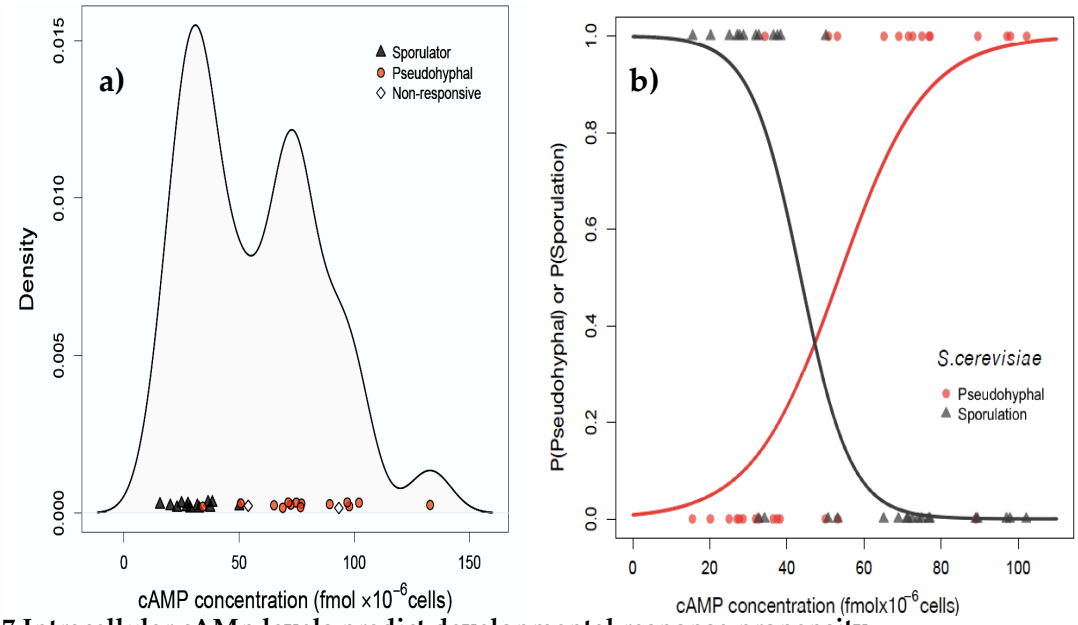

Figure 7 Intracellular cAMp levels predict developmental response propensity
(a) Intracellular cAMP concentrations exhibit bimodal distribution in *S. cerevisiae* strains. **(b)** Logistic regressions of sporulation (black) and pseudohyphal growth (red) on intracellular levels of cAMP. The model predicts the trade-off between pseudohyphal growth and sporulation ($p<0.01$)

Differential expression of key genes under nitrogen limitation

The cAMP-PKA signaling affects the activity of a number of transcription factors and nuclear proteins to regulate developmental responses. I measured gene expression levels for a set of loci involved in cAMP signaling, sporulation and pseudohyphal growth (CYR1, FLO8, FLO11, GPA2, IME1, IME2, PHD1, RAS2, RME1, SOK2, TPK1, TPK2, TPK3) (Table 3). Gene expression was measured relative to ACT1, under nitrogen limiting conditions. All but three loci (PHD1, RAS2, and TPK2) exhibited significant differential expression between strains with strong and poor pseudohyphal responses ($p<0.05$) (Figure 8). There was at least a two-fold difference in expression for genes with differential expression values. The highest difference was observed for FLO11 followed by IME1 and RME1.

Table 3 Key genes affecting pseudohyphal and sporulation responses

p values indicate significance in differential expression levels for corresponding genes between pseudohyphal at sporulator strain

Gene Name	Gene Function	Responses affected	p values
Cyr1	Adenylate cyclase	Sporulation and pseudohyphal growth	1.19E-05
Flo8	Transcription factor	Pseudohyphal growth	3.69E-04
Flo11	Cell surface protein	Pseudohyphal growth	2.99E-05
Gpa2	G protein alpha subunit	Pseudohyphal growth	1.74E-05
Ime1	Transcription factor	Sporulation	5.04E-06
Ime2	Serine/Threonine protein kinase	Sporulation	3.35E-05
Phd1	Transcription factor	Pseudohyphal growth	0.26
Ras2	GTP-binding protein	Sporulation and pseudohyphal growth	0.28
Rme1	Zinc-finger protein	Sporulation and pseudohyphal growth	2.89E-05
Sok2	Transcription factor	Sporulation and pseudohyphal growth	1.01E-05
Tpk1	Serine/Threonine protein kinase	Pseudohyphal growth	1.46E-03
Tpk2	Serine/Threonine protein kinase	Pseudohyphal growth	0.19
Tpk3	Serine/Threonine protein kinase	Pseudohyphal growth	1.43E-02

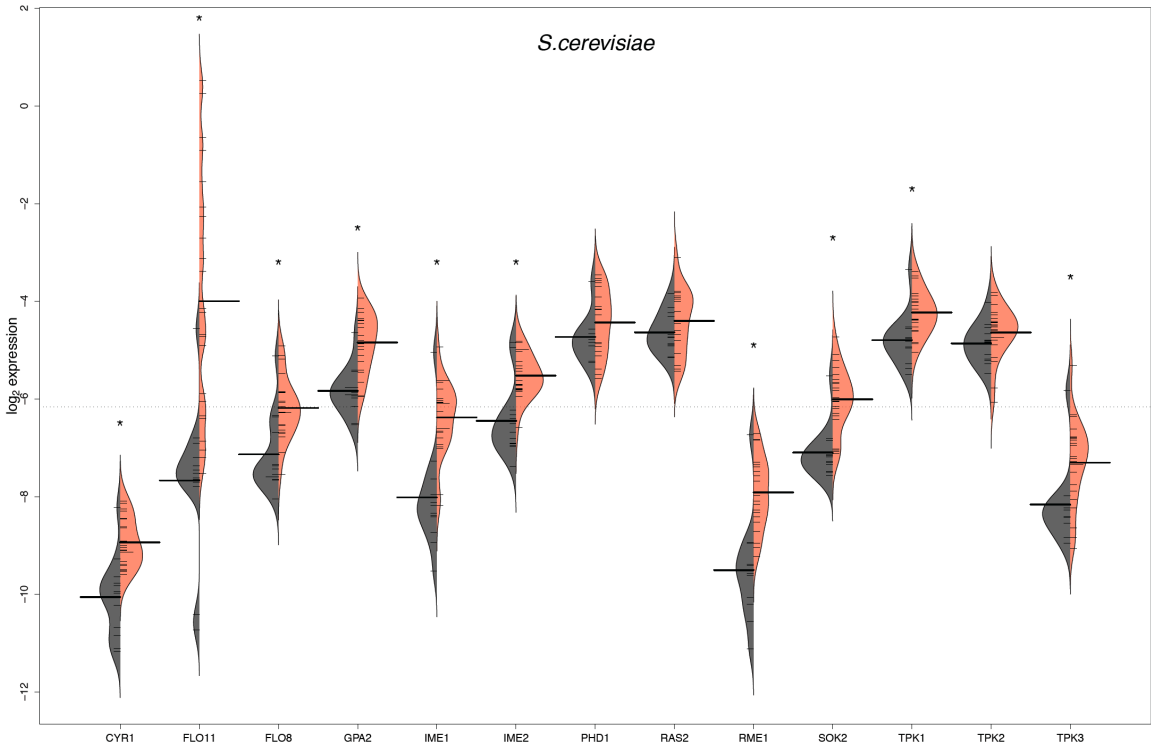

Figure 8 Differential expressions of key genes under nitrogen limitation
Joint histograms of expression levels for key genes in pseudohyphal and non-pseudohyphal *S. cerevisiae* strains. Black: Non-pseudohyphal, Red: Pseudohyphal
The black bars in the histograms indicate the mean. (*) marks the significant differences at ($p<0.05$)

Levels of cAMP signaling and RME expression capture variation in sporulation

To investigate how sporulation efficiency varies with cAMP concentrations and RME1 expression I carried out regression analyses for sporulation as a function of intracellular cAMP levels and RME1 expression. Sporulation efficiency was inversely correlated with intra-cellular cAMP measurements with a coefficient of determination of $R^2 = 0.62$ (Figure 9a). Similarly, RME1 expression also showed a negative correlation with sporulation efficiency, $R^2 = 0.50$ (Figure 9b). A multiple regression model in which sporulation was regressed jointly on cAMP concentrations and RME1 expression levels

captures majority of the between-strain variation in sporulation ($R^2 = 0.78$) (Figure 9c).

Coefficients for analyses were significant at ($p < 0.01$).

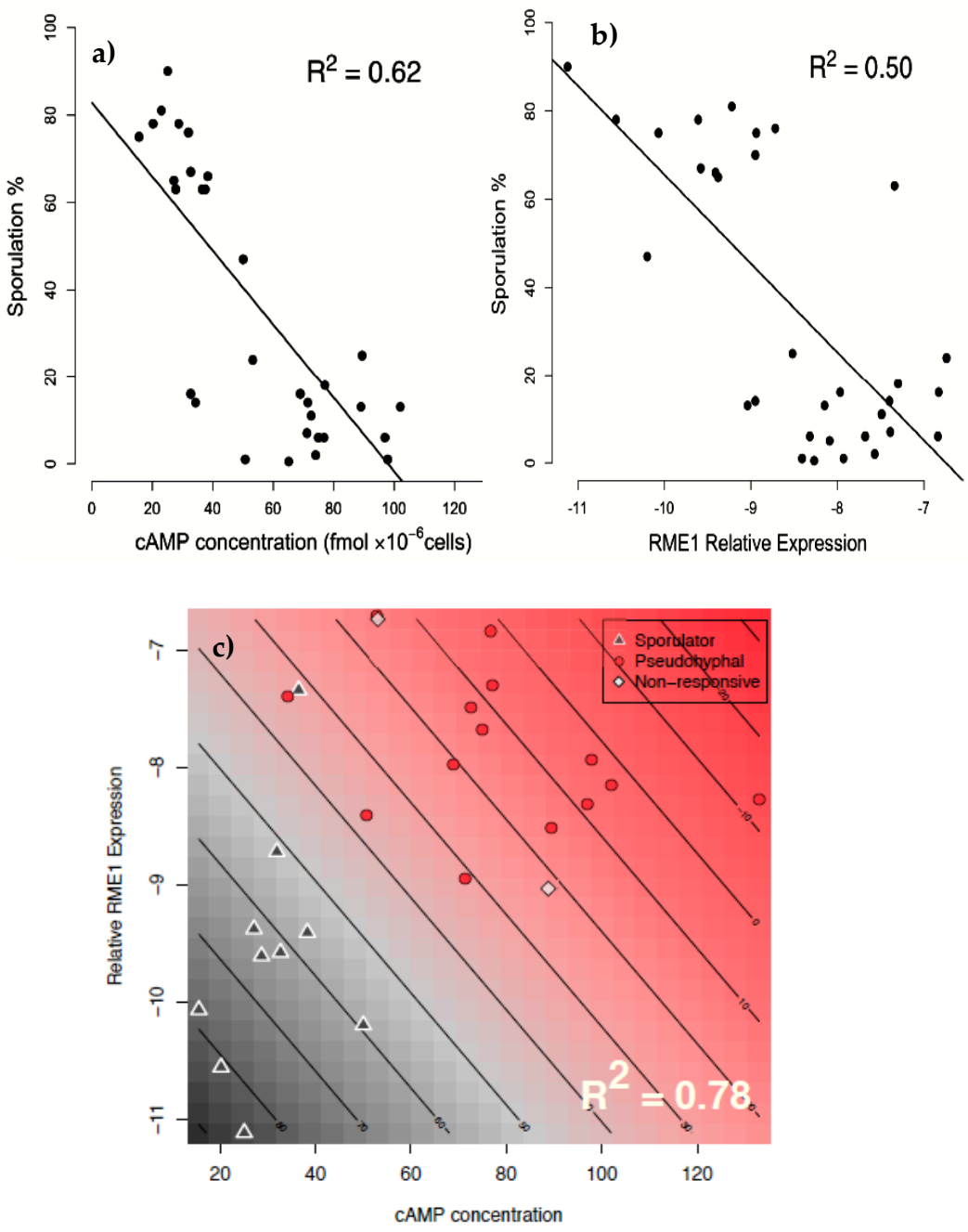

Figure 9 Levels of cAMP signaling and RME expression capture variation in sporulation
Intracellular cAMP and RME1 expression levels capture between strain variation in sporulation efficiency. **(a,b)** Sporulation efficiency inversely correlates with cAMP levels and RME1 expression measured in *S. cerevisiae* strains under nitrogen limiting conditions (SLAD-1%). **(c)** A joint regression on cAMP levels and RME1 expression explains most of variation in sporulation efficiency.

Co-segregation of sporulation and non-sporulation alleles

The genetic data were collected by genotyping 33 strains for indels in RME1 and RAS2 promoters that were identified as sporulation QTLs [100,101]. I found that of the fifteen sporulator strains 12 had insertions in position -308 of RME1 and three had an insertion in a poly-A stretch upstream of RAS2. I also detected a combination of deletions in -308 of RME1 and poly-A stretch in 14 of the 18 pseudohyphal strains (Figure 10). I describe these combinations of QTLs to be correlated with sporulation efficiency, and hence the phenotypic trade-off.

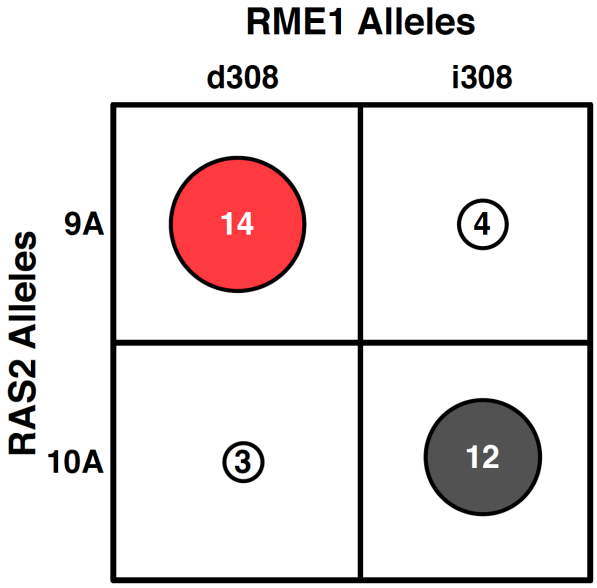

Figure 10 Sporulation efficiency segregates with allelic variation
Particular indel combinations at promoter regions of RME1 and RAS2 are strongly correlated with developmental phenotypic trade-off in *S. cerevisiae.* Numbers of pseudohyphal strains with non-sporulation alleles are circled in red, and sporulation strains with sporulation alleles are in black. Thewhite circles show strains that do not have the allele combinations.

2.4 Discussion

Life-history trade-offs are important for fitness of a population; however, molecular mechanisms underlying life-history trade-offs have remained elusive. In this study I present signaling, expression and genetic evidence that the cAMP-PKA signaling underpins a life-history trade-off in *S. cerevisiae*. The highly conserved cAMP-PKA pathway has been indicated in numerous studies as a possible molecular mechanism mediating life-history traits and trade-offs, including growth vs. reproduction[96,102]. For this study I dissect the correlation between pseudohyphal growth and sporulation at the molecular level by investigating the coordinative role of cAMP-PKA signaling in the phenotypic trade-off between pseudohyphal growth and sporulation.

The cAMP-PKA signaling is an important regulator of cellular and organismal functions. In yeast, it promotes growth and development, such as pseudohyphal growth while inhibiting stress-induced responses, such as sporulation [35,38]. The myriad functional roles of this highly conserved signaling pathway in yeast are well-studied. By exerting effects on numerous molecular elements, such as transcription factors and kinases, the cAMP-PKA signaling pathway modulates and fine-tunes multiple levels of molecular interactions that are manifested in phenotypes important for the fitness of an organism.

In *S. cerevisiae*, pseudohyphal growth and sporulation are two alternative life-history strategies to nitrogen limitation[35]. Nitrogen deficiency in the presence of glucose triggers pseudohyphal growth, which has been hypothesized to be a foraging mechanism [97]. Conversely, presence of a non-fermentable carbon source triggers

sporulation, which is defined as meiosis followed by spore formation. These complex traits are governed by intricate molecular activities that feed into unusually large promoters of two key loci leading to pseudohyphal growth and sporulation responses. FLO11 encodes for a cell-surface glycoprotein important for invasiveness and adhesiveness [42]. IME1, on the other hand, is the principal gene for entry into meiosis (Figure 5) [58]. The cAMP-PKA signaling contributes significantly to regulation of these important genes in alternative modes mediating pseudohyphal growth and sporulation.

Magwene et.al. has previously observed an inverse correlation between pseudohyphal growth and sporulation in *S. cerevisiae*, and defined a life-history trade-off between the two survival strategies[91]. In this study, using a larger strain panel, the striking negative correlation between pseudohyphal growth and sporulation was also detected (Figure 6).

The cAMP-PKA pathway critically regulates these alternative developmental traits. The pivotal components and effectors of the cAMP-PKA pathway in yeast developmental responses are well characterized. Numerous studies have shown that elevated cAMP-PKA signaling activity as well as exogenous cAMP increases pseudohyphal response, and conversely, increased cAMP-PKA activity leads to decreased sporulation efficiency (Figure 5) [36]. Thus, based on the existing knowledge in the literature, I reasoned that strains efficient in pseudohyphal growth should have higher cAMP concentrations. The bimodal cAMP distribution in which cAMP levels were significantly higher in pseudohyphal strains compared to non-pseudohyphal ones supported the hypothesis (Figure 7). The considerable match between cAMP

concentrations and distribution of strains for pseudohyphal response further suggests that proficiency for pseudohyphal growth varies with cAMP levels. The joint logistic regression of probability to sporulate and to undergo pseudohyphal differentiation on cAMP levels also shows that the propensity of a strain to undergo pseudohyphal growth or sporulation can be dictated by intracellular cAMP levels. The distinctive cAMP concentrations in pseudohyphal and non-pseudohyphal isolates indicate that cAMP is a likely critical molecular modulator for these alternative life-history strategies. This is the first study to show that levels of a secondary messenger play a decisive function in molecular regulation of a trade-off between principle life-history traits.

Upregulation of genes in the cAMP-PKA pathway as well as increased expression levels of its effectors dictating pseudohyphal growth and sporulation addresses how intimately the cAMP-PKA signaling is linked to the trade-off (Figure 8). Positive correlation between RME1 expression levels and pseudohyphal growth provides a glimpse on transcriptional underpinnings of the trade-off [91]. Since the cAMP-PKA pathway transcriptionally coordinates pseudohyphal growth, regulatory differences at the transcriptional level should evolve between pseudohyphal and non-pseudohyphal strains. The higher expression of genes involved in cAMP synthesis, such as CYR1 and GPA2 reflects the directionality of correlation between pseudohyphal growth and cAMP levels. Similarly, elevated expressions of TPK1 and TPK3 (PKA catalytic subunits important for mitotic growth) emphasize the important role of the pathway in transcriptional regulation of pseudohyphal growth, and hence, in turn, in the trade-off.

In addition to components of the cAMP-PKA signaling, major elements down-stream of the pathway critically regulating pseudohyphal response also show divergent transcriptional activity. Although differential regulation of FLO11 was anticipated between pseudohyphal and non-pseudohyphal strains, increased gene expression levels of key transcriptional factors (FLO8, RME1 and SOK2) directly targeted by cAMP-PKA pathway for regulation of FLO11 provide a convincing evidence on how cAMP might effect the trade-off directly[39,94,103]. Surprisingly, on the other hand, IME1 and IME2, key mediators of sporulation, also display significant increase in expression levels in pseudohyphal strains. While pivotal function of these loci for meiosis and spore formation is well-characterized, recent studies have revealed that these loci also carry out additional roles in transmitting the nutrient signals to pseudophyphal growth beside sporulation, and thus serving as a critical point in life-history decisions[104,105]. Altogether, the significant correlation between cAMP levels, gene expression and pseudohyphal growth strongly suggest a decisive and mechanistic role for the cAMP-PKA signaling in the trade-off between pseudohyphal response and sporulation (Table 3).

Strong and consistent relationship between RME1, cAMP levels and sporulation is predicted given the function of the two variables in pseudohyphal growth. RME1 is an important contributor and a good predictor of the phenotypic trade-off between pseudohyphal growth and sporulation and RME1 expression captures a modest proportion of variation in sporulation with a significant coefficient of determination (R^2 = 0.5) (Figure 9a)[91]. As discussed in previous paragraphs, sporulation efficiency

declines with higher cAMP levels in *S. cerevisiae*. Compared to RME1 expression, a linear regression of sporulation on cAMP concentrations explains a slightly higher proportion of the variation ($R^2 = 0.62$) (Figure 9b). This is probably due to the effects of cAMP-PKA signaling on several elements governing the response. Sporulation is a complex trait regulated by numerous factors, and yet nonetheless a multiple regression analysis of sporulation proficiency on both RME1 expression and cAMP levels captures a significant and substantial amount of variation in sporulation ($R^2 = 0.78$) (Figure 9c). This result strongly suggests that RME1 and cAMP-PKA signaling is mechanistically linked to the phenotypic trade-off.

Genetic determinants controlling sporulation efficiency are in accord with the observed differences in propensities for pseudohyphal and sporulation responses. Indels in RME1 and RAS2 promoters have been identified as sporulation QTLs important for the decision to initiate meiosis and sporulation [100,101]. RME1 is a zinc-finger protein that represses IME1 (Initiator of MEiosis) while promoting mitosis by upregulating expression of G1-cyclins [106]. RAS2 encodes a small G-protein that stimulates pseudohyphal growth by activating the cAMP-PKA pathway, and inhibits sporulation by increasing cAMP levels via activation of adenylate cyclase (Cyr1)[9]. Co-segregation of polymorphisms at these critical loci complements the expression and signaling data, and presents a genetic basis for the phenotypic trade-off between pseudohyphal growth and sporulation.

3. Functional Evolution of cAMP-PKA Signaling in *Saccharomyces sensu stricto*

3.1 Introduction

Cells largely rely on signaling pathways to assess and respond to environmental changes. External stimuli bombarding cells are internalized as signals that are processed and integrated into decision-making mechanisms. Coordinative and complementary actions of signaling cascades form regulatory networks mediating cellular processes important for survival. Various growth and developmental strategies relevant to different life-styles and diverse niches help organisms endure harsh/suboptimal environmental conditions. Although signaling components and pathways governing these diverse strategies are broadly maintained throughout evolution, signaling interactions reconfigure and tune cellular responses in the context of life-style and preferred habitat.

The cyclic AMP-Protein Kinase A (cAMP-PKA) signaling is a conserved pathway and a prominent regulator of growth, proliferation, development, and differentiation from yeast to man[5]. The cAMP-PKA signaling pathway interacts with other signaling pathways and steers pivotal developmental responses to changing stimuli[15,30]. In the model eukaryote, *Saccharomyces cerevisiae*, the cAMP-PKA signaling pathway governs pseudohyphal growth and sporulation in response to nutrient availability (Figure 11) [35]. Nitrogen limitation is a trigger for both responses; however, in the presence of a fermentable carbon source, such as glucose, the cAMP-PKA signaling promotes pseudohyphal response and suppresses sporulation in diploid yeast cells[36].

Pseudohyphal differentiation is characterized by cell elongation, physical attachment of mother-daughter cells, and ability to adhere and invade substrates[97]. Nitrogen deficiency, on the other hand, induces sporulation on respiratory substrates, such as acetate[57]. During this process cells exit vegetative growth and undergo meiosis followed by spore formation.

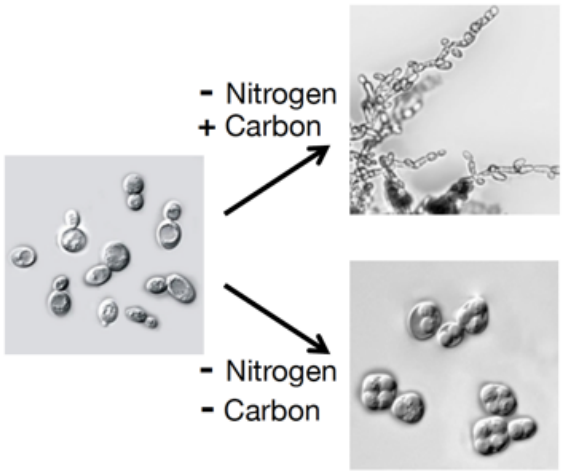

Figure 11 Pseudohyphal and sporulation responses in Saccharomyces
Upon nitrogen depletion, Saccharomyces yeast undergoes pseudohyphal differentiation in the presence of a fermentable carbon source, glucose; or sporulates when a non-fermentable carbon, acetate, is available.

The mechanism and the critical role of the cAMP-PKA signaling in these inversely correlated developmental traits have been well-studied in *S. cerevisiae*. Pseudohyphal differentiation is proportional to levels of cAMP, a ubiquitous secondary messenger. Adenylate cyclase synthesizes cAMP upon activation via two G-protein systems (Ras2 and Gpa2) when glucose is sensed and metabolized. Increased cAMP levels trigger PKA as cAMP molecules to bind to the regulatory subunit (Bcy1) and release catalytic subunits (Tpk1, Tpk2, Tpk3). Activated PKA engages phosphodiesterases (Pde1 and Pde2) as well as GTPase activating proteins (Ira1 and

Ira2) in a negative feedback loop that controls cAMP concentrations [36]. Also, the active PKA targets a multitude of proteins whose activity affects pseudohyphal response by converging on the unusually large promoter of FLO11[42]. In *S. cerevisiae,* this locus encodes a cell-surface glycoprotein essential for pseudohyphal switching[41]. PKA subunits antagonistically affect transcriptional regulation of FLO11, which is complexed and critical for the response. Tpk1 and Tpk3 repress the response by affecting transcription factors such as Sok2 and Phd1[36]. Tpk2, on the other hand, activates expression of FLO11 by affecting Flo8 and Sfl1[39]. In addition to the cAMP-PKA signaling, the MAPK cascade also upregulates FLO11 expression through transcription factors Ste12 and Tec1 (Figure 12a). Furthermore, under pseudohyphal conditions the cAMP-PKA signaling, together with Sch9, derepresses transcriptional factors Rim15 and Msn2 to activate the general stress response mechanism that also converges on the FLO11 promoter (Figure 12b) [32,107]For this work, I investigated functional interactions and evolution of the cAMP-PKA signaling as a regulator of pseudohyphal response using three *Saccharomyces* species.

S. cerevisiae, *S. paradoxus* and *S. bayanus* species complex (*S. bayanus* from here forward) experience different selection forces in diverse niches they occur (Figure 13). Widely utilized *S. ceresivise* has been isolated from cultivated and natural habitats, and extensively employed in human activities. Its closest relative *S. paradoxus*, however, has been mostly isolated from wild environments, such as oak tree michrohabitats, and has not been specialized for human activities. *S. bayanus* has diverged much earlier from a common ancestor and is known to be more tolerant to nutrient stress[63,66,108].

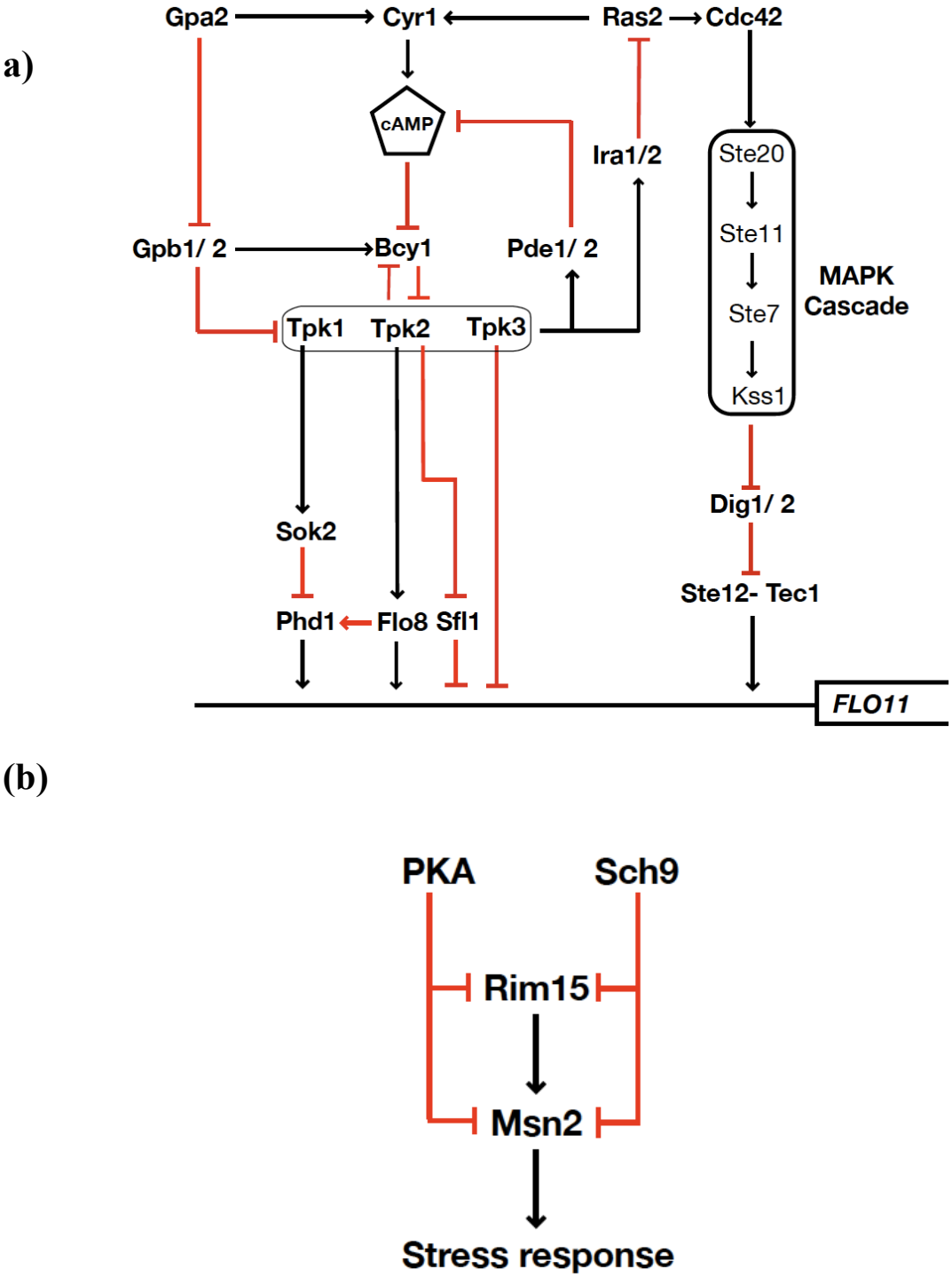

Figure 12 The cAMP-PKA signaling regulates pseudohyphal differentiation at multiple levels Upon nitrogen limitation, the cAMP-PKA signaling mediates multiple factors for transcriptional regulation of FLO11, an adhesin required for pseudohyphal growth in *S. cerevisiae*. MAPK cascade also converges on FLO11 promoter for regulation of the response. **b)** When nutrients are sub-optimal, PKA and Sch9 derepresses general-stress response mechanism in *S. cerevisiae* that also modulates pseudohyphal growth.

Comparative studies on *Saccharomyces* species have revealed how rewiring contributes to functional flexibility of gene networks underlying adaptive changes in survival responses. For example, the presence of several different states of GAL network across the lineage is reflective of ecological settings in terms of galactose utilization. Another example of regulatory divergence has been observed in differential transcriptional regulation of membrane proteins that are associated with altered stress responsiveness of *S. paradoxus*, indicating a different selective pressure experienced by this species[109]. A recent comparative work has also shown that binding site divergence for Ste12 and Tec1 (pseudohyphal response transcription factors) in *S. cerevisiae, S.bayanus* and *S. mikatae* underlies extensive changes in gene expression regulation, and also captures the role of functional rewiring and flexibility in adapting to distinct ecological niches [110].

In this chapter, I compare and discuss phenotypic, signaling, and genetic data that show that the cAMP-PKA signaling regulates pseudohyphal response and sporulation in an alternative fashion in *S. bayanus*. Although these species can be found in sympatry, they display different physiologies, and functional flexibility of the cAMP-PKA signaling is very likely to contribute to distinct differences observed in growth and survival strategies between these organisms. More importantly, significant rewiring of a central signaling pathway, such as the cAMP-PKA signaling, in such closely related species so as to function antagonistically provides formidable inferences about general principles on rewiring of regulatory systems.

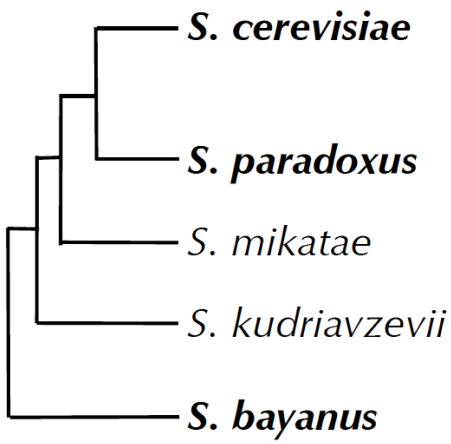

Figure 13 Closely related *Saccaromyces* species
Saccharomyces species inhabit a broad range of environments and exhibit different physiologies despite the short estimated-divergence time between lineages (5-20 mya).

3.2 Materials and Methods

Strains, media, and phenotyping:

Strains: Strains phenotyped for this study are given in (Table 4) *S. bayanus* homozygous null mutants were generated on NCYC365 background using KanMX4 deletion-cassette [111]with the standard PEG/LiAc protocol modified at the heat shock step, which was performed at 37°C for 45 m . The generated mutants, listed in (Table 5), were confirmed with PCR and Sanger sequencing using primers given (Table 6). **Media & Phenotyping:** Strains were grown overnight in YPD to a density of $2x10^7$ cells/ml. The cells were then washed twice in sterile water and 10^6 cells were transferred to agar plates. Pseudohyphal growth was assayed using a modified SLAD medium (SLAD-1%) consisting of 0.17% YNB − AA/AS, 1% dextrose, 50 μM ammonium sulfate, and 2% Noble agar [97]. For drug treatments plates were supplemented with the indicated concentrations of cAMP (Enzo), 8-Bromoadenosine 3',5'-cyclic monophosphate [8-Br-cAMP] (Sigma), 3-isobutyl-1-methylxanthine [IBMX] (Sigma), H-

89 (Sigma), MDL 12,330A [MDL] (Sigma), and 2′-5′-Dideoxyadenosine [ddAdo] (Santa Cruz). For phenotyping, *S. cerevisiae* and *S. paradoxus* were incubated at 30°C, and *S. bayanus* strains were incubated at room temperature (RT). The strains were scored for pseudohyphal growth as presence or absence of cellular projections at the colony edges, and the response was evaluated qualitatively as increase (**+**), decrease (**-**), and no change (∅) at 72 h. Images were collected using Leica Stero microscope.

Intracellular cAMP measurements

Intracellular cAMP concentrations were determined for strains grown in liquid SLAD-1% media for 4 hr at 30°C for *S. cerevisiae* and *S. paradoxus*, and at RT for *S. bayanus*. 34 μL of cell culture was fixed in 300 μL 1 M n-butanol saturated formic acid, and lysed by four freeze-thaw cycles in dry ice. The formic acid was then evaporated using a Speed Vac Concentrator (Savant Instruments, Hickville, NY). cAMP levels were quantified following the Non-Acetylation EIA protocol for the cAMP Biotrak EIA kit (RPN225, GE Healthcare).

Table 4 List *Saccharomyces* strains surveyed (Chapter 3)

Pseudohyphal growth scored as absence(0) or presence (1), sporulation efficiencies were measured percentages

Strain	Strain Name	Origin	Pseudohyphal	Sporulation
PMY 361	YPS138	Oak	0	49.76
PMY 362	N-43	Oak	0	28.40
PMY 363	CBS432	Oak	1	0.00
PMY 364	Y7	Oak	0	64.41
PMY 365	Q89.8	Oak	0	79.06
PMY 366	Q74.4	Oak	0	81.32
PMY 367	Z1	Oak	0	72.44
PMY 368	DBVPG6304	*Drosophila*	1	69.23
PMY 369	N-44	Oak	0	0.00
PMY 370	N-17	Oak	1	81.94
PMY 371	Y6.5	Oak	0	27.95
PMY 372	Q95.3	Oak	0	90.13
PMY 373	Q96.8	Oak	0	67.79
PMY 374	Y8.1	Oak	0	80.12
PMY 375	A4	Oak	1	0.00
PMY 376	N-45	Oak	0	35.09
PMY 377	CBS5829	Soil	1	0.00
PMY 378	Q32.3	Oak	1	66.04
PMY 379	S36.7	Oak	0	0.00
PMY 380	LD7	Oak	1	60.76
PMY 381	A12	Oak	0	25.57
PMY 382	IFO1804	Oak	0	51.50
PMY 383	DBVPG4650	Guano	1	0.00
PMY 384	Q59.1	Oak	0	60.39
PMY 385	Z1.1	Oak	0	63.58
PMY 386	Q31.4	Oak	1	0.00
PMY 387	KPN3828	Oak	1	0.00
PMY 388	UFRJ50791	*Drosophila*	0	96.59
PMY 389	T21.4	Oak	0	80.13
PMY 390	Q62.5	Oak	0	87.18
PMY 391	Y9.6	Oak	0	71.75
PMY 392	Y8.5	Oak	0	71.52
PMY 393	KPN3829	Oak	1	0.00
PMY 394	UFRJ50816	*Drosophila*	0	25.45
PMY 395	UWOPS91-917.1	Flux, *Myoporun*	0	0.00

Strain	Strain Name	Origin	Pseudohyphal	Sporulation
PMY 640	NCYC365	Apple juice	1	24.70
PMY 641	NCYC2578	Turbid beer	1	3.35
PMY 642	CBS7001	*Mesophylax adopersus*	1	0.00
PMY 643	YJM520	Fermenting apple juice	1	0.00
PMY 644	YJM519	Pear juice	0	0.00
PMY 645	NCYC509	*Ribes nigrum* juice	1	0.00
PMY 660	GL 222	Unknown	1	0.00
PMY 661	GL 274	Unknown	0	44.23
PMY 668	VKM Y-361	Czech wine	0	0.00
PMY 669	NRRL Y-969	Unknown	1	0.00
PMY 670	VKM Y-1146	Grape berries	1	1.36
PMY 734	NBRC539	Unknown	1	0.00
PMY 735	NCYC114	Unknown	1	41.62
PMY 736	NBRC10558	Must of soft fruit	0	0.00
PMY 911	ZP555	Oak	1	0.00
PMY 912	ZP556	Oak	0	93.56
PMY 937	NCYC686	Spoiled Coca-Cola	1	0.00
PMY 938	NCYC762	Palm wine	0	47.08
PMY 939	NCYC1322	Irish Brewery	0	1.34
PMY 940	NCYC1323	Australian Brewery	1	0.00
PMY 941	NCYC1324	Scottish Brewery	0	0.00
PMY 942	NCYC1341	British Brewery	0	0.00
PMY 943	NCYC3066	Fermenting Saurkraut	0	87.92
PMY 944	NCYC3359	Cider apples	1	0.00
PMY 947	NCYC1326	British Brewery	0	0.00
PMY 948	NCYC1342	British Brewery	1	0.00
PMY 949	NCYC965	British Brewery	1	0.00
PMY 952	NCYC966	British Brewery	1	0.00
PMY 953	NCYC967	British Brewery	1	0.00
PMY 955	NCYC969	British Brewery	0	0.00
PMY 956	NCYC984	European Brewery	0	0.00
PMY 957	NCYC985	European Brewery	1	0.00
PMY 958	NCYC986	European Brewery	1	0.00
PMY 959	NCYC987	European Brewery	1	0.00
PMY 969	NCYC988	European Brewery	0	0.00
PMY 970	NCYC989	European Brewery	1	66.53

Table 5 List of primers for confirmation-PCR

Primer	Sequence (5'-3')
KanB	CTG CAG CGA GGA GCC GTA AT
KanC	TGATTT TGA TGA CGA GCG TAA T
SB_BCY1_AC	CCCGCTCTCGCTCAGAGGA
SB_BCY1_DC	CCCCTGCGAAATCGTGTCCATCG
SB_DIG1_AC	CCTGTGCGTGAGTTTGTGTGTTTG
SB_DIG1_DC	GGCAGGAAAATGTTCAGAC
SB_FLO8_AC	GGCAATTCTTTGATAGCAAC
SB_FLO8_DC	CCCGTTCCTATTGTGGAAGTCG
SB_FLO11_AC	GGCATTGCTTACAATAGGTTCCTG
SB_FLO11_DC	GGTATTGTTGAACACGGAATGG
SB_GPA2_AC	CGTCCGTGTACAGCTTCAAG
SB_GPA2_DC	GGGTATAGGACATTCACTG
SB_GPB1_AC	CCGTTCGTTAGAATGTTC
SB_GPB1_DC	GGTCTGCGTTCAGGACTTAAC
SB_GPB2_AC	CCGTTCGTTAGAATGTTCAC
SB_GPB2_DC	CCTGCGCGCAATCTGTAATC
SB_IRA2_AC	CGCAGCGTCGGTACACCCTG
SB_IRA2_DC	CTTCAACAGAAGAATGCTCCC
SB_MSN2_AC	ACACGAACACAGGTACCAC
SB_MSN2_DC	GTATCTTACTAGTTACAGGC
SB_PDE1_AC	GGTAGGTTCGATTACTCATGG
SB_PDE1_DC	GGCTCAGCCAGATGCTTTGTCC
SB_PDE2_AC	GACCAAGAAATGGTATTTGC
SB_PDE2_DC	CCTCTGCATAATTCGACCTC
SB_PHD1_DC	CGGACGCTATTCTTGGCAGC
SB_PHD1_DC	CGCTTAGGTGTGGGAGATCCG
SB_RAS1_AC	GCATATGGTACCCGTACTGG
SB_RAS1_DC	GATTCGTGACAATTTCCC
SB_RAS2_AC	GCGTGTCCAACCAAGATTGG
SB_RAS2_DC	GGCATGCGACACCAGGAC
SB_RIM15_AC	GCCTTGGGAGAATACGTGAAAC
SB_RIM15_DC	CCTCGGTCTTAAACATGCATATG
SB_SCH9_AC	GCCTATCTTAAATCTCCTCC
SB_SCH9_DC	CGCATCGGAAAGACCAGCC
SB_SFL1_AC	CCATCGTGCATCTCCGGC
SB_SFL1_DC	CCCATGGACATCCCAGTC
SB_STE7_AC	GGGAATATTCAATGCAGT
SB_STE7_DC	GGGAAACCATTCAAGTATGG
SB_STE12_AC	GGTATATCAGAAACAAGTCG
SB_STE12_DC	TGTAGTTGGTATCACCGC
SB_TEC1_AC	CGGCTCGAAGAATTTCTC
SB_TEC1_DC	GGCTAAATTCATGCAAATCC
SB_TPK1_AC	CCCAAGGCTGACCTCACC
SB_TPK1_DC	GCGTGAAAGCTTCTCATCTCC
SB_TPK2_AC	CTACATTCGGCCTATTAAAC
SB_TPK2_DC	GTGACTCGGCACTTCCTGTCG
SB_TPK3_AC	CGCTGGCTCATGTATCCAT
SB_TPK3_DC	GCCAGGTTCGCTTTTAGC

Table 6 List of *Saccharomyces* mutants

Species	Strain	Genotype	Reference
S. bayanus	PMY 1392	*gpa2Δ*:: kanMX	This study
	PMY 1394	*ste7Δ*:: kanMX	This study
	PMY 1396	*flo8Δ*:: kanMX	This study
	PMY 1442	*dig1Δ*:: kanMX	This study
	PMY 1445	*ras2Δ*:: kanMX	This study
	PMY 1446	*tec1Δ*:: kanMX	This study
	PMY 1448	*tpk1Δ*:: kanMX	This study
	PMY 1450	*ste12Δ*:: kanMX	This study
	PMY 1455	*pde1Δ*:: kanMX	This study
	PMY 1459	*tpk3Δ*:: kanMX	This study
	PMY 1460	*sch9Δ*:: kanMX	This study
	PMY 1461	*pde2Δ*:: kanMX	This study
	PMY 1462	*bcy1Δ*:: kanMX	This study
	PMY 1479	*tpk2Δ*:: kanMX	This study
	PMY 1480	*rim15Δ*:: kanMX	This study
	PMY 1481	*ras1Δ*:: kanMX	This study
	PMY 1482	*gpb1Δ*:: kanMX	This study
	PMY 1483	*gpb2Δ*:: kanMX	This study
	PMY 1484	*ira2Δ*:: kanMX	This study
	PMY 1596	*msn2Δ*:: kanMX	This study
	PMY 1600	*flo11Δ*:: kanMX	This study
	PMY 1603	*phd1Δ*:: kanMX	This study
	PMY 1606	*sfl1Δ*:: kanMX	This study
S. cerevisiae	PMY 485	*gpa2Δ*:: kanMX	Lorenz & Heitman, 1997
	PMY 487	*ste12Δ*:: kanMX	Lorenz & Heitman, 1997
	PMY 491	*flo8Δ*:: kanMX	Liu, *et.al.*, 1996
	PMY 498	*ira2Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 499	*ras2Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 501	*tec1Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 506	*tpk1Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 507	*tpk2Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 508	*tpk3Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 746	*pde1Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 748	*flo11Δ*:: kanMX	Drees, et.al., 2005
	PMY 750	*pde2Δ*:: kanMX	Drees, *et.al.*, 2005
	PMY 827	*ste7Δ*:: kanMX	Lorenz & Heitman, 1997
	PMY 843	*phd1Δ*:: kanMX	Lorenz & Heitman, 1998
	PMY 861	*bcy1Δ*:: kanMX	Pan & Heitman, 1999
	PMY 982	*rim15Δ*:: kanMX	Pan & Heitman, 1999
	PMY 1039	*msn2Δ*:: kanMX	Pan & Heitman, 1999
	PMY 1048	*sch9Δ*:: kanMX	Lorenz, et.al., 1999
	PMY 1064	*sfl1Δ*:: kanMX	Pan & Heitman, 2002

qPCR and gene expression measurements

Total RNA was isolated from independent cell cultures grown in SLAD-1% media using hot acid/phenol protocol. cDNA was generated using an iScript cDNA synthesis kit (BioRad). Gene expression was measured by quantitative RT-PCR using the iQ SYBR Green kit (BioRad). qPCR reactions were carried out on a Rotor-Gene 6000 real-time cycler (Corbett Research). ACT1 was used as a "housekeeping" gene to normalize expression values. For the strains and growth conditions ACT1 exhibits relatively small inter-strain and -species variation and thus is a good basis for normalization. Primers for the qPCR reactions are listed in Table 2. 4. Primers were synthesized by Sigma-Aldrich. Relative expression was calculated as $\log_2(E_R^{Ct} - E_T^{Ct})$, where E_R and E_T are the amplification efficiencies of the reference (ACT1) and the target gene; and Ct is the measured cycle thresholds for each gene during the qPCR reaction [99].

3.3 Results

Distinct developmental and signaling responses to nutrient limitation

A survey of pseudohyphal growth and sporulation in *Saccharomyces* species showed different response propensities to nitrogen limitation. Phenotypic scoring conducted on strains of *S. paradoxus* and *S. bayanus* from a wide range of habitats indicated a lack of strong trade-off between pseudohyphal growth and Sporulation that was observed for *S. cerevisiae*. *S. paradoxus* isolates showed a weak inverse correlation between the responses while distinct trade-off pattern in the plots was completely absent in *S. bayanus* (Figure 14). Previous work has reported that *S. cerevisiae* strains

with various levels of pseudohyphal response exhibit less than 40% sporulation efficiency whereas non-pseudohyphal isolates were proficient in Sporulation by greater than 40% [91]. The break down of the weak phenotypic trade-off between pseudohyphal differentiation and sporulation in *S. paradoxus* was as follows: four of 11 pseudohyphal strains were good sporulators and 8 of 24 Non-pseudohyphal strains were poor sporulators. The *S. bayanus* strain panel, on the other hand, had only 6 strains with proficient sporulation, two of which were also good at pseudohyphal growth with another 20 strains (total 22 pseudohyphal strains). There were 14 Non-pseudohyphal isolates including the remaining four proficient sporulators. The low number of sporulators in the *S. bayanus* panel is a reminder that this species is well adapted to nutrient stress, especially nitrogen deficiency.

To see how intracellular cAMP levels correlate with pseudohyphal and Sporulation in *S. paradoxus* and *S. bayanus* I measured cAMP concentrations in the two strain-panels under nitrogen limiting conditions. When compared to *S. cerevisiae*, intracellular levels of cAMP were much lower in the two species, ranging between 4.0-22.8 fmol/10^6cells for *S. paradoxus*, and 3.3 -18.3 fmol/10^6cells for *S. bayanus.* Although low concentrations were predicted, the narrow distribution of cAMP levels for both *S. paradoxus* and*S. bayanus* was unanticipated (Figure 15).

Exogenous cAMP inhibits pseudohyphal growth in *S. bayanus*

Based on these data, I reasoned that low concentrations of cAMP in *S. paradoxus* and *S. bayanus* was not sufficient to induce pseudohyphal, and increasing the cAMP levels would enhance the response as it does in *S. cerevisiae*. To test this hypothesis a

pseudohyphal and a non-pseudohyphal strain were phenotyped under nitrogen-limiting conditions in the presence of cAMP at various concentrations. Non-pseudohyphal *S. cerevisiae* and *S. paradoxus* isolates displayed a typical pseudohyphal response in which colonies had rough edges and increased agar invasiveness. Similarly, pseudohyphal strains also exhibited an increased response upon cAMP treatment. However, exogenous cAMP treatment, strikingly, inhibited pseudohyphal differentiation in *S. bayanus*. Not only was the cAMP treatment ineffective in inducing the response in a non-pseudohyphal *S. bayanus* isolate but, surprisingly, also suppressed pseudohyphal differentiation in a pseudohyphal *S. bayanus* strain (Figure 16).

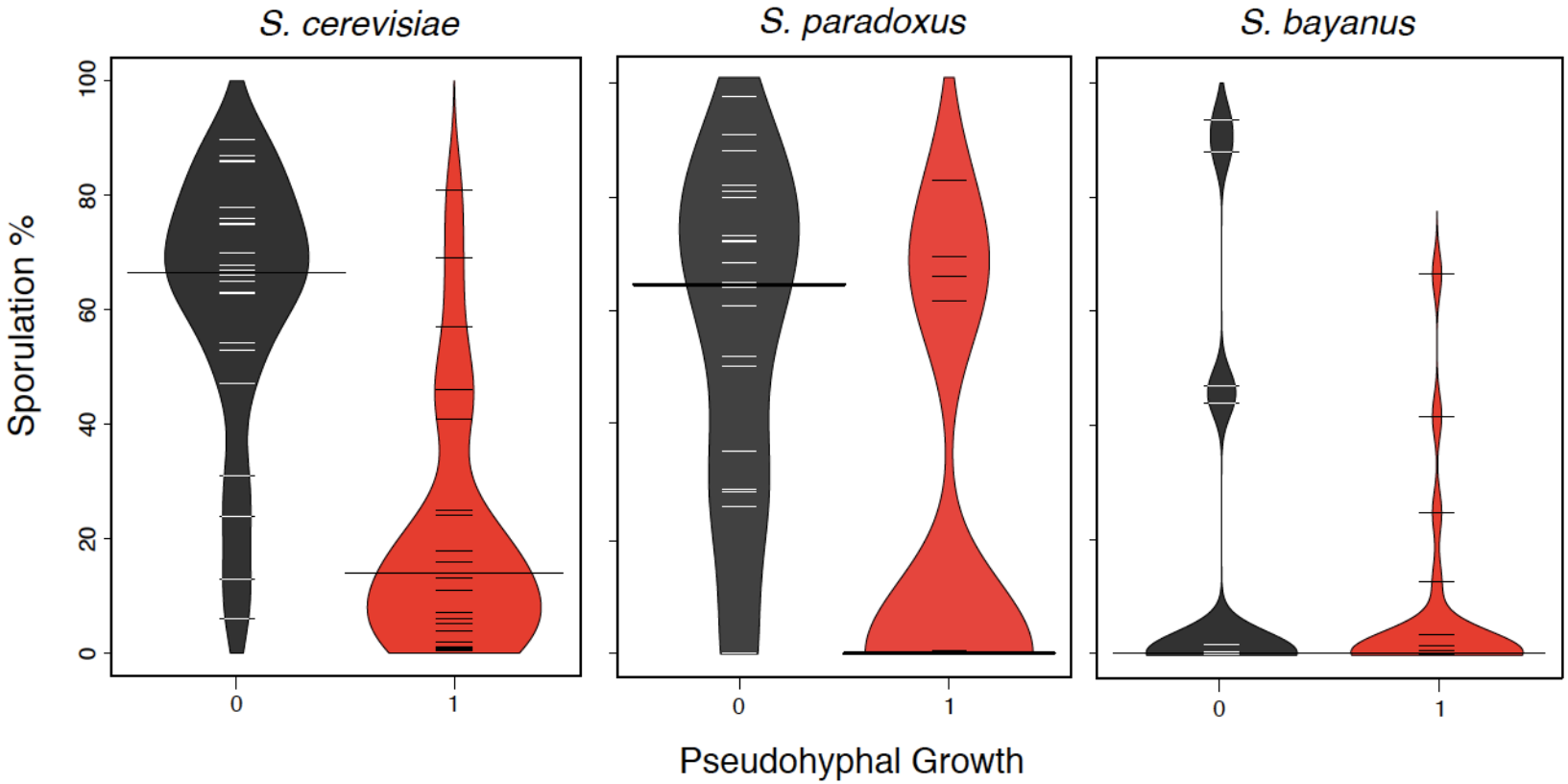

Figure 14 Distinct correlations of phenotypic responses in *Saccharomyces*
Bean plots indicate a strong and a weak trade-off in *S. cerevisiae*(49 isolates) and *S. paradoxus* (35 isolates), respectively. The plots for *S. bayanus* (39 isolates) suggests the marked inverse correlation between pseudohyphal and sporulation is absent. "0"/Black: Non-Pseudohyphal strains"1"/Red : Pseudohyphal strains. The thick line is drawn the median.
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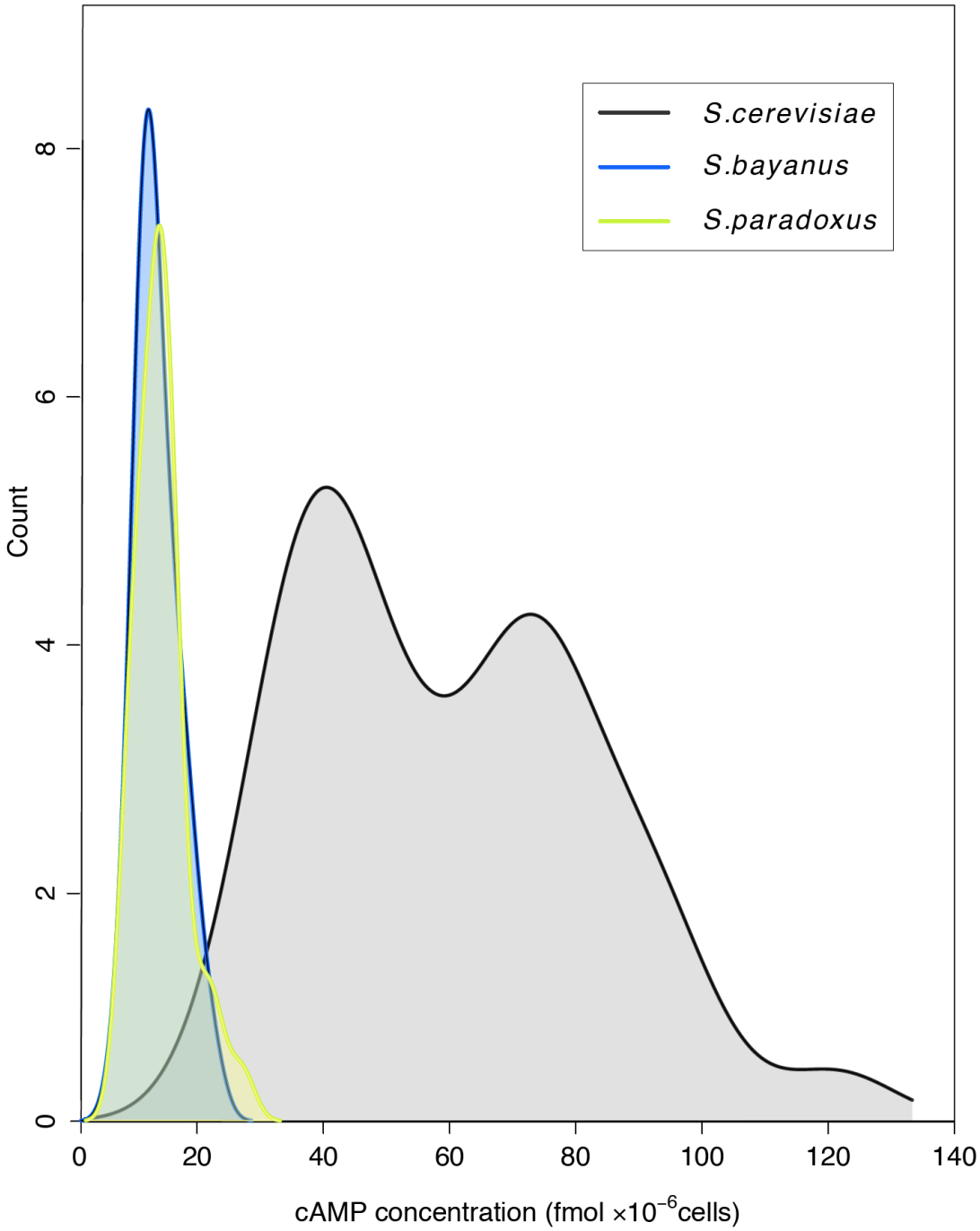

Figure 15 Different intracellular cAMP levels in *Saccharomyces*
Intracellular cAMP concentrations show different distributions in *S. cerevisiae*, *S. paradoxus* and *S. bayanus*. The levels of cAMP (in liquid SLAD-1%, 4h)are much higher in *S. cerevisiae* strains and significantly higher in pseudohyphal strains. Intracellular cAMP levels are low in *S. paradoxus* and *S. bayanus*, and do not show bimodal distributions.

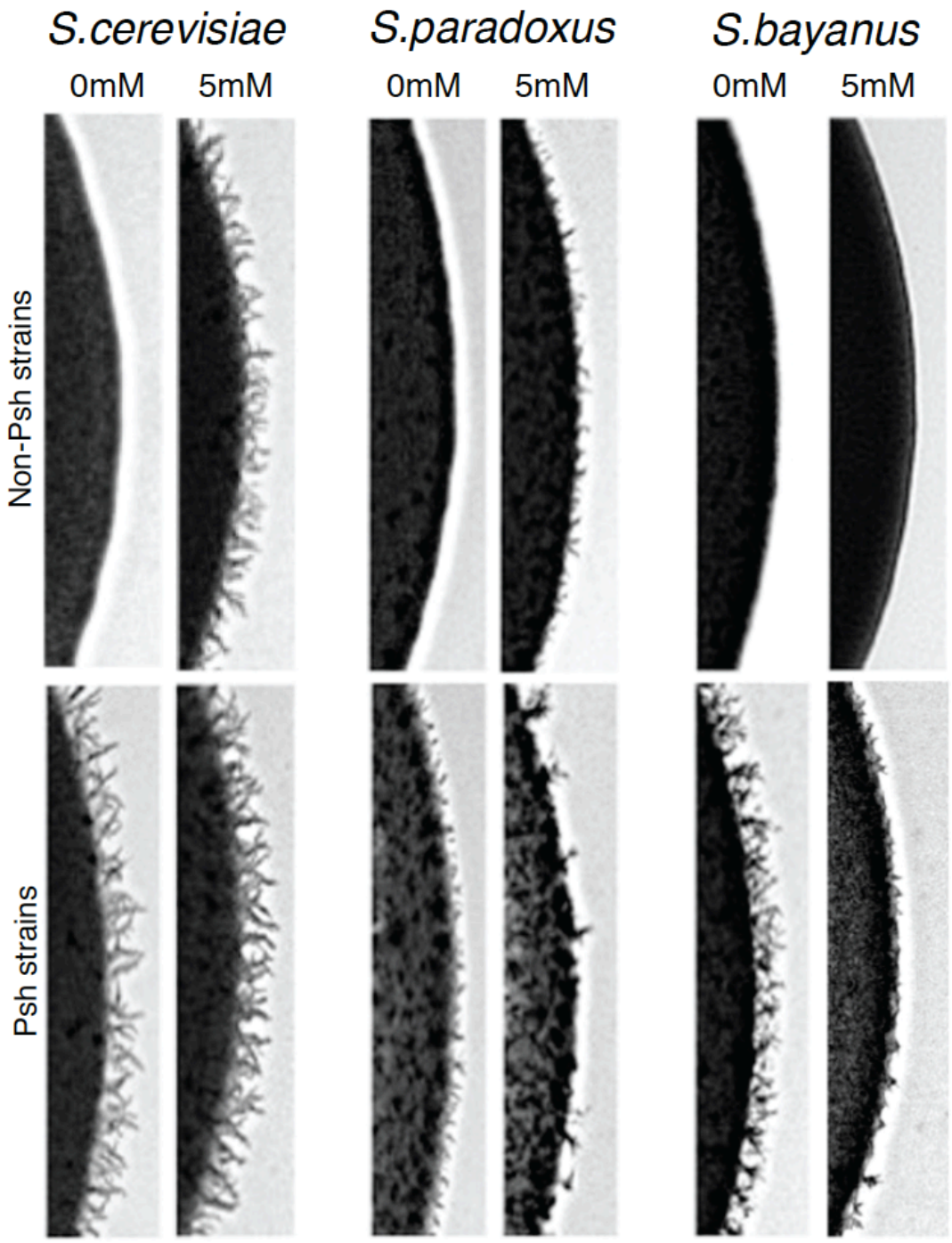

Figure 16 Exogenous cAMP inhibits pseudohyphal growth in *S. bayanus*
Pseudohyphal responses for a pseudohyphal (Psh) and a non-pseudohyphal (Non-Psh) strain of each indicated species were assayed on SLAD-1% plates with or without cAMP at 72 h. The cAMP treatment promotes pseudohyphal growth in *S. cerevisiae* (Σ1278b) and *S. paradoxus*(PMY368) but not in *S. bayanus*(PMY640).

To rule out that this was a strain/ concentration-specific result the rest of the strains in the *S. bayanus* panel were also phenotyped for pseudohyphal growth in the presence of various cAMP concentrations. The observations were consistent with that of the exogenous cAMP inhibited nutrient-induced pseudohyphal developmet in *S. bayanus* (Figure 17). In order to further explore the surprising effect cAMP had in *S. bayanus*, pseudohyphal response was also scored in the presence of a cAMP analog 8-Br-cAMP (more membrane permeant and resistant to PDE degredation), a PDE inhibitor (IBMX) that would generate an increase in intracellular cAMP levels, and a PKA inhibitor (H-89), as would be the case under insufficient cAMP levels (Figure18). Effects of 8-Br-cAMP (500 μM) and a low concentration of IBMX (1 mM) were the same as the results of the exogenous cAMP treatment in all three species. A higher concentration of IBMX (3 mM), however, led to a loss of the response in *S. cerevisiae* and *S. paradoxus*, probably due to complete shut down of the feedback mechanism controlling cAMP levels [112]. PKA inhibitor H-89 (50 μM) had no discernible effects on pseudohyphal growth in *S. cerevisiae* and *S. bayanus* , however inhibited the response in *S. paradoxus*.

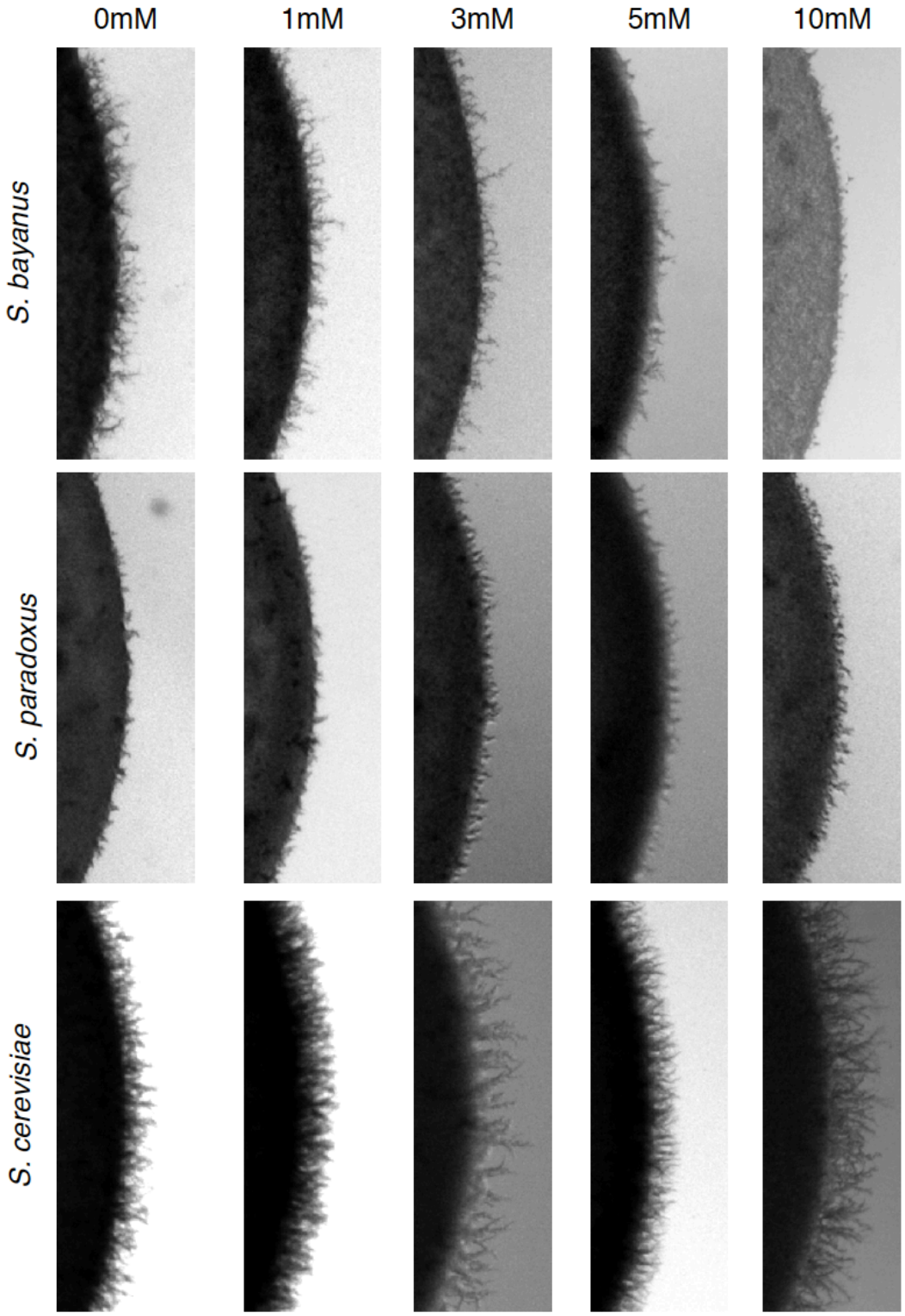

Figure 17 Different concentrations of exogenous cAMP inhibits differentiation in *S. bayanus*
The effects of exogenous cAMP on pseudohyphal growth are proportional to the strength of the phenotypic response.

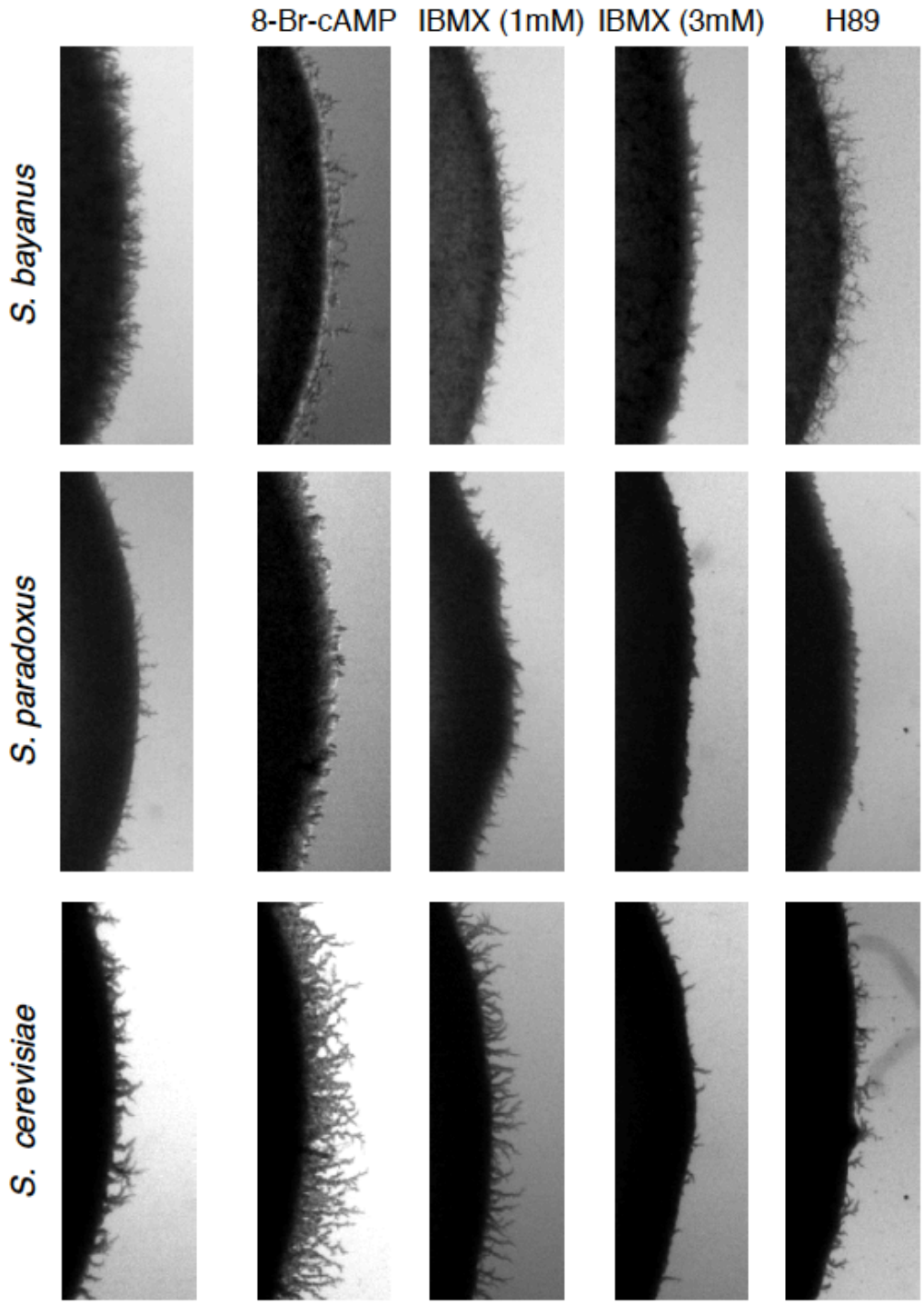

Figure 18 Pharmacological modulations of cAMP levels

Intracellular cAMP levels were modified using indicated drugs in three ***Saccharomyces*** species. Effects of 8-Br-cAMP were similar to exogenous cAMP treatment. Low concentrations of IBMX reflect the effects of increased intracellular cAMP levels but a higher concentration blocks the response likely due to collapse of the cAMP signaling. PKA inhibitor H89 had a strong effect on *S. paradoxus*

MAPK functions similarly in *S. cerevisiae* and *S. bayanus* pseudohyphal response

To dissect genetic components and outline a possible mechanism for the surprising effect that increased cAMP levels have on pseudohyphal growth key loci regulating the response in *S. cerevisiae* were deleted in *S. bayanus*. Since MAPK is another critical signaling cascade mediating the response I also examined whether this signaling contributes to pseudohyphal growth similarly in *S. cerevisiae* and *S. bayanus*[37]. The disruption of activators, *ste7Δ*, *ste12Δ*, and *tec1Δ*, caused smooth colony edges, and deletion of an inhibitor, *dig1Δ*, increased the response (Figures 19 and 20).

The cAMP-PKA pathway is required for *S. cerevisiae* and *S. bayanus* pseudohyphal response

Yet, unlike MAPK mutants, effects of gene deletions in the cAMP-PKA pathway grouped in three categories when compared with *S. cerevisiae*: 1) mutants with same effects 2) mutants with parallel effects 3) mutants with opposite effects (Table 7). The first category included *gpa2Δ*, *pde2Δ*, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* that are upstream of the pathway, and data from these mutants indicated that the cAMP-PKA signaling function in the same manner, relative to *S. cerevisiae*, in *S. bayanus* for pseudohyphal growth Deletion of GPA2 and TPK2 led to a total loss of response, while deletion of TPK1 and TPK3, known inhibitors of pseudohyphal switch in *S. cerevisiae,* exhibited a strong pseudohyphal growth on SLAD-1% (Figure21). In addition, deletion of PDE2, and hence disruption of the feedback did not cause a significant change, the pde2*Δ* strain showed a comparable pseudohyphal growth relative to the wild type (Figure 22).

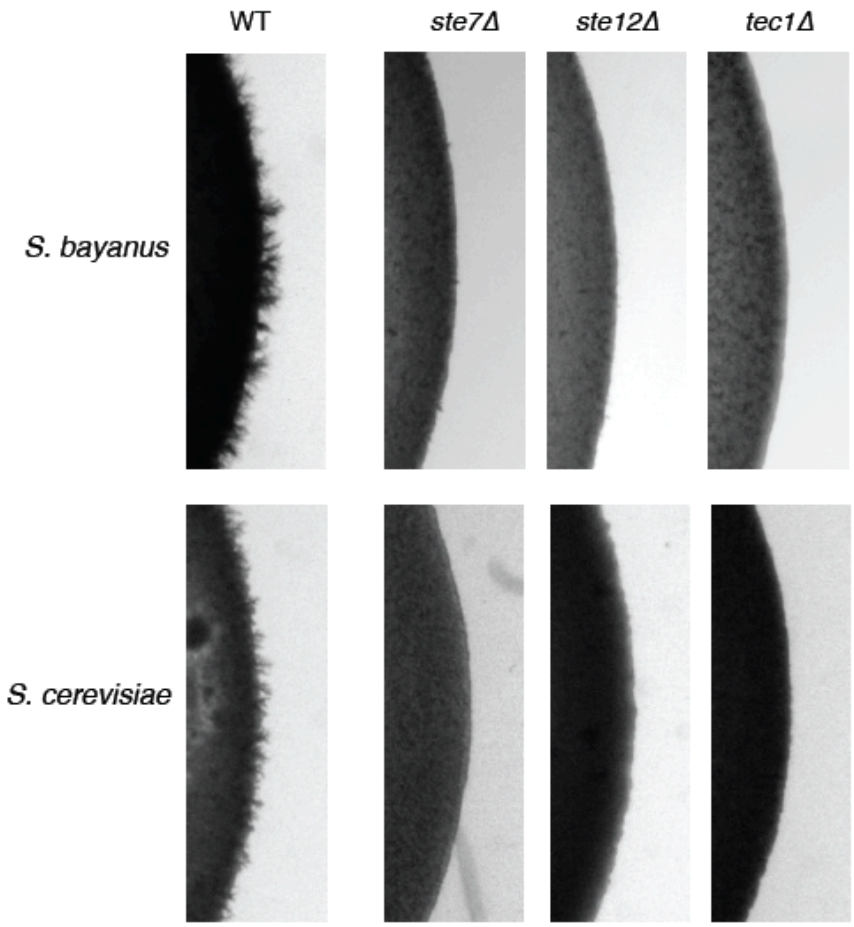

Figure 19 Disruption of MAPK signaling eliminates pseudohyphal growth in *Saccharomyces* cerevisiae and *S. bayanus,* indicating that the cascade regulates the response positively in the two species. Mutants are on Σ1278b and PMY640 backgrounds for *S. cerevisiae* and *S. bayanus*, respectively.

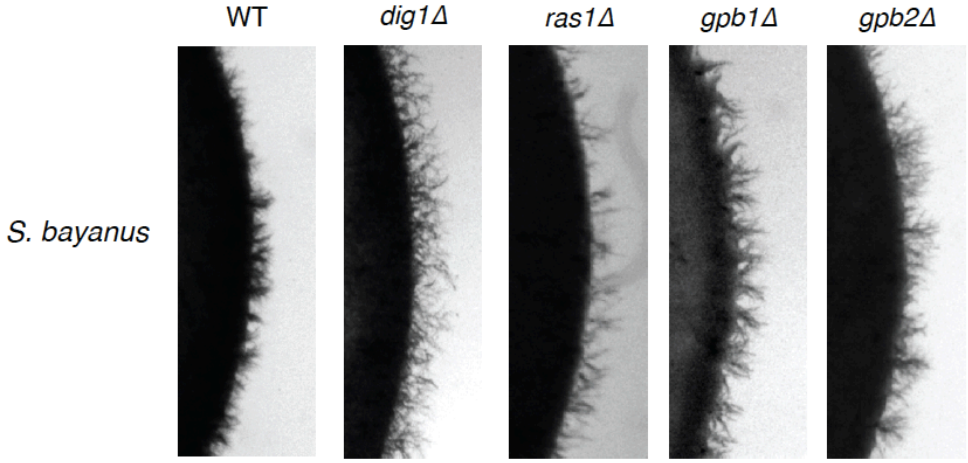

Figure 20 *S. bayanus* mutants with similar pseudohyphal responses relative to *S. cerevisiae* Pseudohyphal growth in these mutants compared to published observations in *S. cerevisiae*

Table 7 Categories of cAMP-PKA signaling disruptions in *S. bayanus*

The pseudohyphal response was evaluated qualitatively as increase (+), decrease (-), and no change (∅) at 72 h. 1=Similar effects 2=Parallel effects 3= Opposite effects

Category	Gene	*S. cerevisiae*	*S. bayanus*
1	*gpa2*	-	-
1	*pde2*	∅	∅
1	*phd1Δ*	-	-
1	*sfl1Δ*	-	-
1	*tpk1*	+	+
1	*tpk2*	-	-
1	*tpk3*	+	+
2	*gpb1*	+	∅/+
2	*gpb2*	+	∅/+
2	*ras1Δ*	∅	∅/-
2	*ras2*	-	∅/-
3	*bcy1*	+	-
3	*flo8*	-	+
3	*flo11*	-	∅
3	*ira2*	+	-
3	*pde1*	+	-

Phenotypes of the mutants in the second category were different. Although results were in the predicted direction, observed strength of the response was not as anticipated in these mutants. Ras2 deletion eliminates pseudohyphal growth completely in *S. cerevisiae*, however, in *S. bayanus* a *ras2Δ* mutation exhibited pseudohyphal differentiation and invasiveness. Similar to *ras2Δ*, *ras1Δ* also had a slight decrease in the response (Figure 20 and 21). Likewise, while *S. cerevisiae* literature reports a gain in pseudohyphal response upon GPB1 and GPB2 deletions[113], in *S. bayanus* observed phenotypes for *gpb1Δ* and *gpb2Δ* were comparable with the wild type(Figure 20).

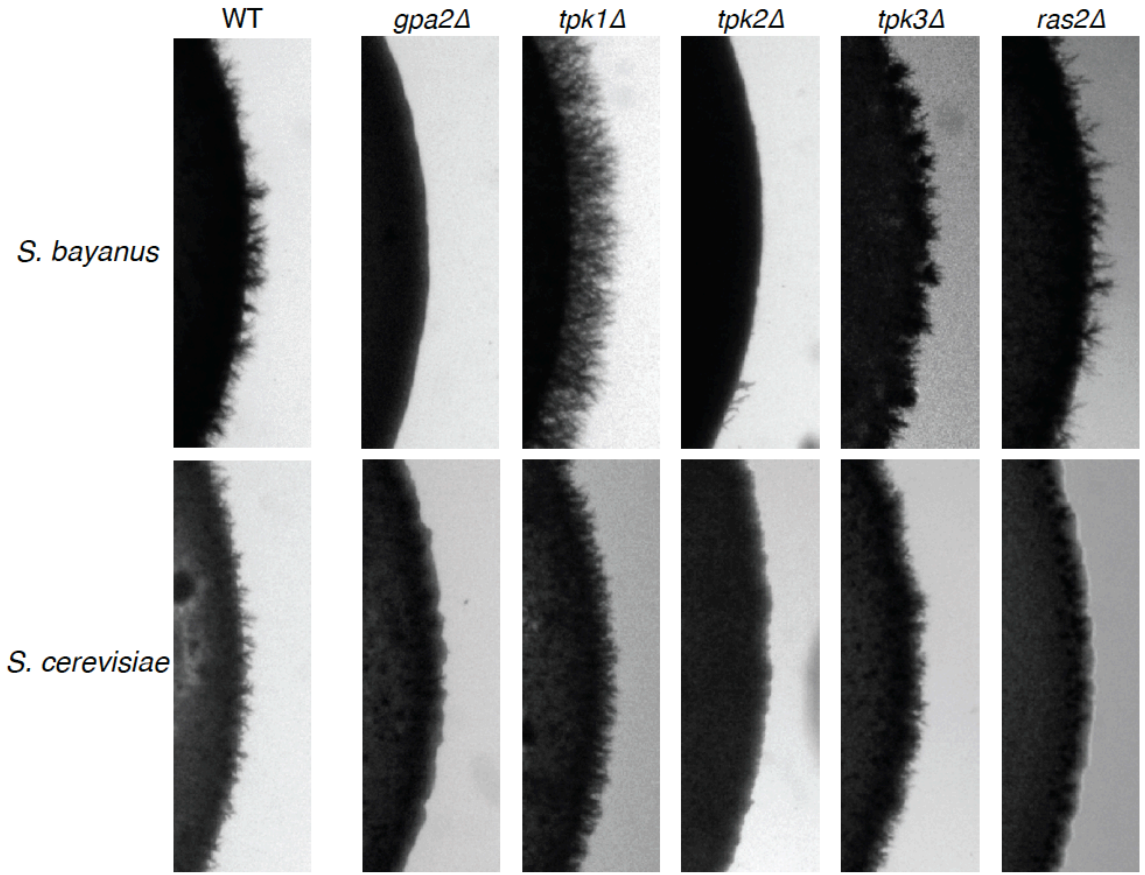

Figure 21 The cAMP-PKA signaling is required for induction of pseudohyphal in *S. bayanus*
Mutants were phenotyped as described in Section 3.2. Mutants are on Σ1278b and PMY640 backgrounds for *S. cerevisiae* and *S. bayanus*, respectively.

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Divergent interactions of the cAMP-PKA signaling regulates pseudohyphal growth antagonistically in *S. bayanus*

The unanticipated responses grouped in the third category were very interesting as they showed a complete contrast to *S. cerevisiae*. The *pde1Δ* and *ira2Δ* mutations, which lead to increased cAMP levels and pseudohyphal growth in *S. cerevisiae*, caused a complete loss of the response in *S. bayanus* (Figure22). Moreover, *BCY1* deletion, which causes a hyperactive PKA and in turns a stronger pseudohyphal response in *S. cerevisiae*[14], surprisingly, resulted in loss of pseudohyphal growth in *S. bayanus* (Figures 23). Another unexpected observation was also that the strain with a deletion mutation for FLO8 was able to undergo pseudohyphal differentiation (Figure 24). Flo8 is a principal transcription activator under the control of cAMP-PKA signaling and is required for upregulation of FLO11 in *S. cerevisiae* [39].

Unlike *flo8Δ*, deletion of the transcription repressor Sfl1 and the transcription activator Phd1, regulated by the cAMP-PKA and known to affect FLO11 expression, exhibited similar phenotypes as observed in *S. cerevisiae* (Figure 24). Strikingly, however, in accord with the *flo8Δ* phenotype, *flo11Δ* mutant, although delayed, did display a pseudohyphal response in *S. bayanus* (Figure 25). Since CYR1 is essential, I further scored pseudohyphal growth for *S. bayanus* mutants with adenylate cyclase inhibitors MDL and ddAdo. Inhibition of this integral element of the cAMP-PKA signaling induced a slight increase in *S. bayanus*, however, suppressed pseudohyphal differentiation in *S. cerevisiae* and *S. paradoxus* (Figure 26). Additionally, ddAdo treatment (300 μM) promoted cellular projections at colony edges in all *S. bayanus*

mutants, except *phd1Δ*. The response was absent event at Day 6, at which point all the strains had a visible increase in pseudohyphal growth (Figure 26).

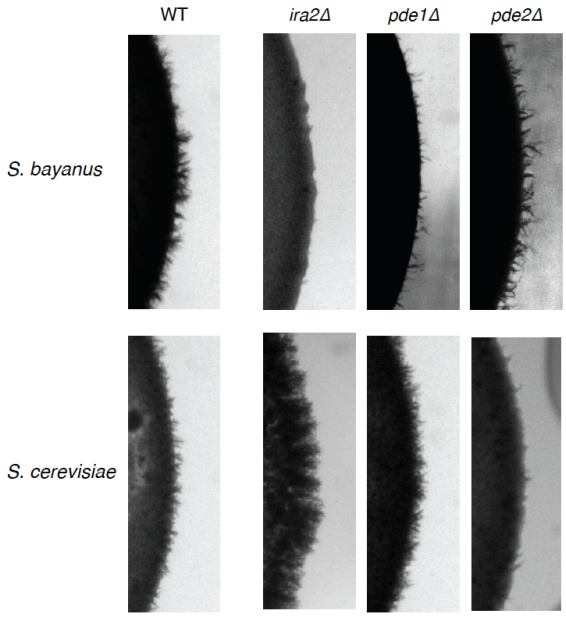

Figure 22 ira2Δ and pde1Δ deletions and divergence
Genetic manipulations increasing the levels of intracellular cAMP, as phenotyped under nitrogen deprivations, led to divergent pseudohyphal responses in *S. cerevisiae* and *S. bayanus*

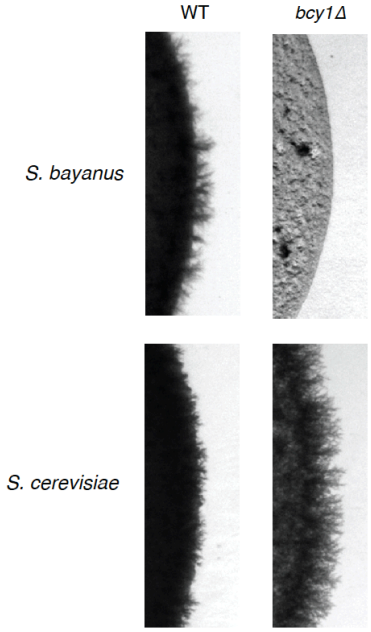

Figure 23 Hyperactivation of PKA and divergence
The *bcy1Δ* mutant causes a complete loss of pseudohyphal differentiation in *S. bayanus,* consistent with effects of increased cAMP levels. The *bcy1Δ* mutant exhibits hyperfilamentation in *S. cerevisiae*

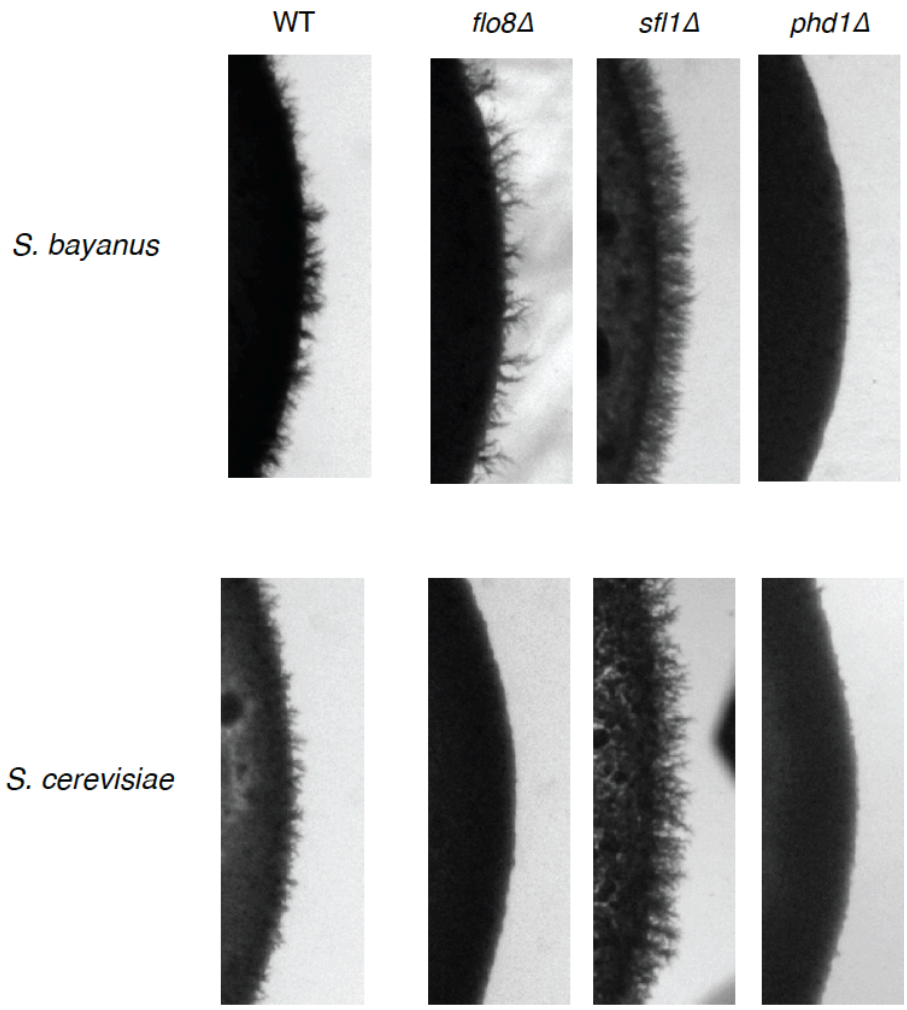

Figure 24 Downstream targets and divergence
The flo8Δ pseudohyphal response is divergent in *S. bayanus,* but the response upon individual deletions of SFL1 and PHD1 is conserved. The cAMP-PKA signaling modulates functions of these transcription factors on FLO11, promoting the response.

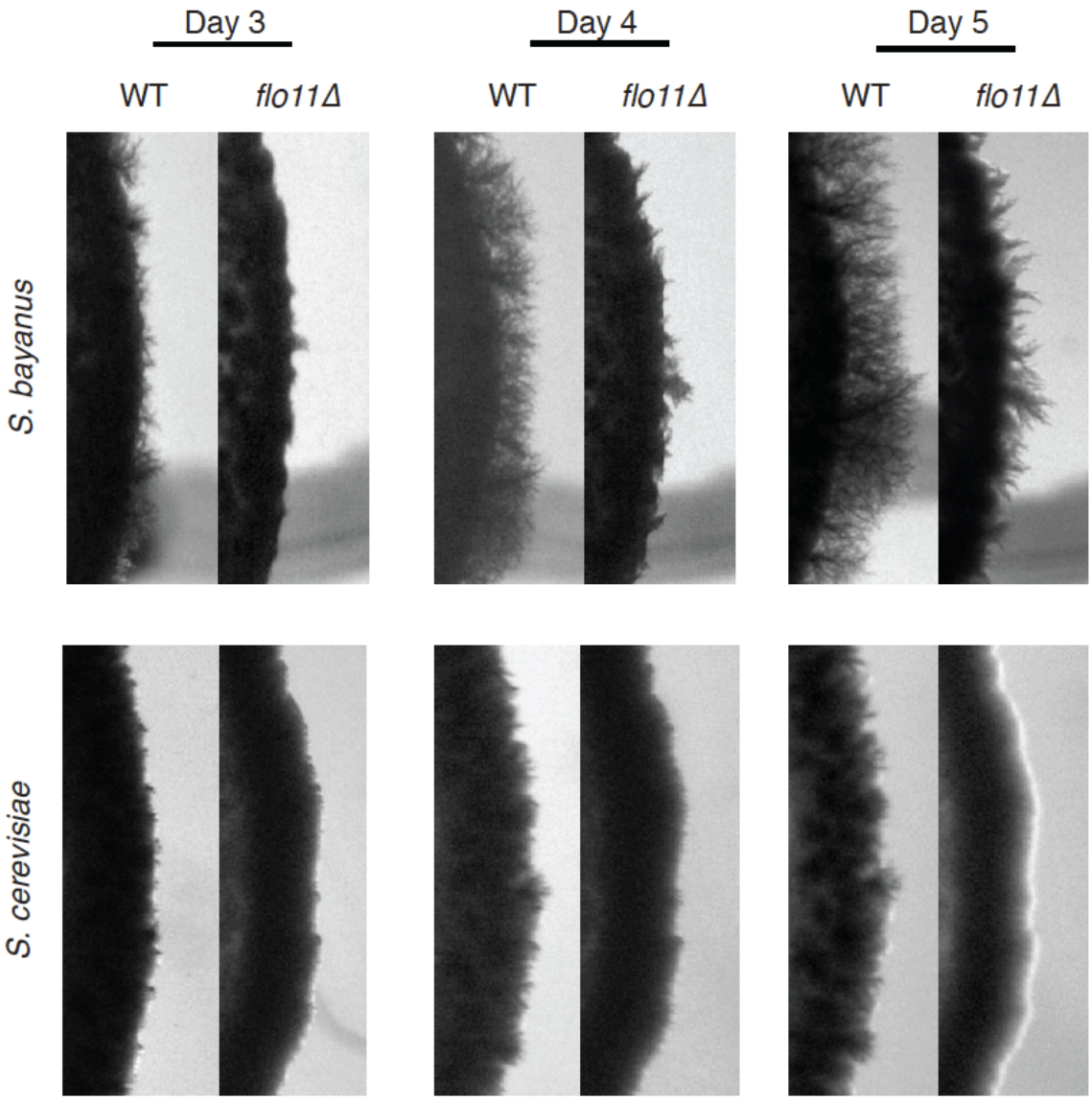

Figure 25 Flo11 is dispensable for pseudohyphal growth in *S. bayanus*.
FLO11 encodes a cell-surface glycoprotein and required for pseudohyphal differentiation in *S. cerevisiae.*

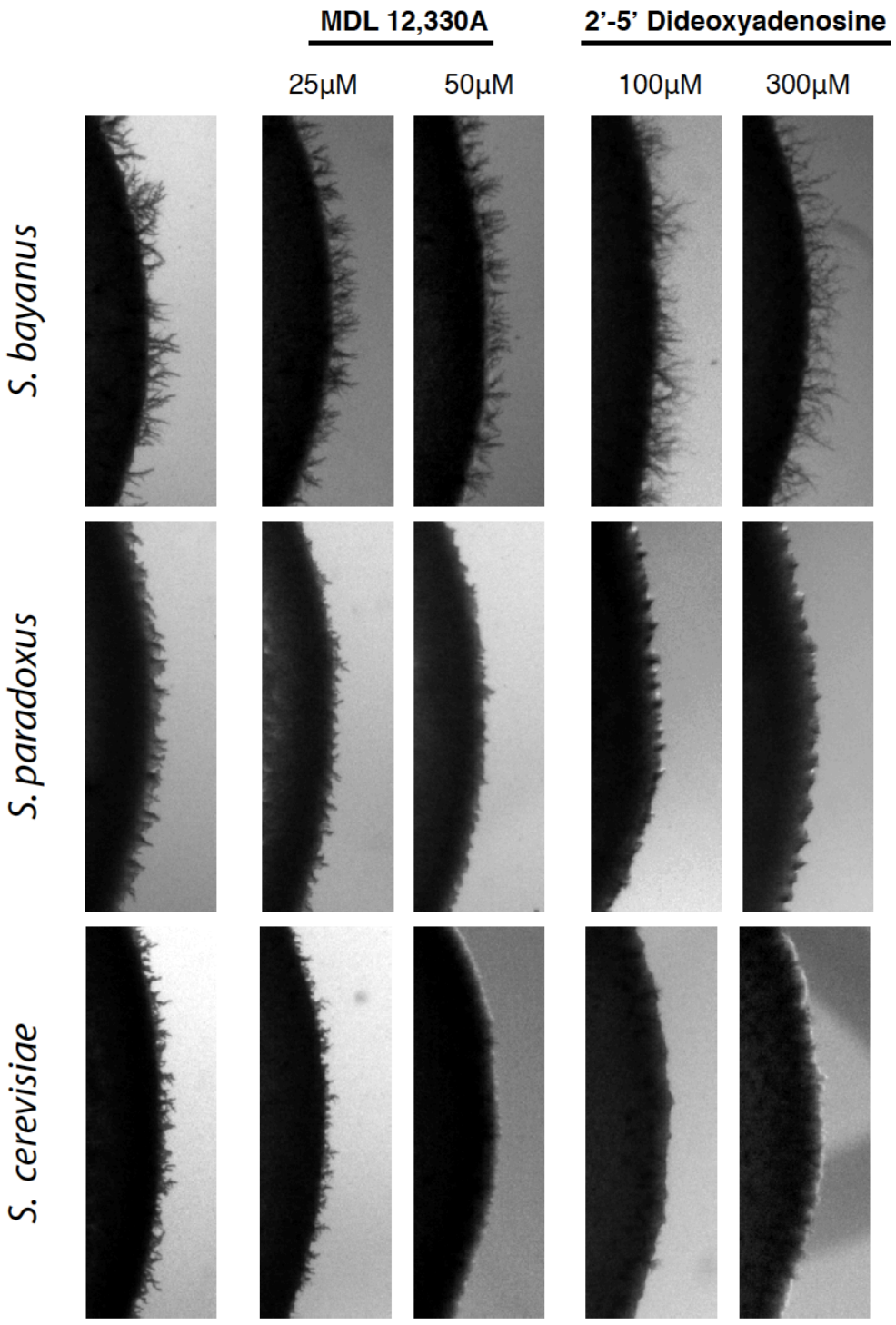

Figure 26Pharmacological inhibition of cAMP synthesis and divergence
MDL 12, 330A and 2′-5′ Dideoxyadenosine inhibit AC activity in different ways. MDL 12,330A prevents the membrane localization of AC, and 2′-5′ Dideoxyadenosine blocks the catalytic domain of AC

Different transcriptional profiles under nitrogen limiting conditions developmental genes

Since the genetic and biochemical data showed that the cAMP-PKA signaling is divergent in regulation of pseudohyphal differentiation in *Saccharomyces* species, this divergence should also be reflected in their transcription profiles under nitrogen-limiting conditions. Expression levels for CYR1, FLO11, FLO8, GPA2, IME1, IME2, RME1, SOK2, STE12, TEC1, TPK1, TPK3 showed significant difference between pseudohyphal and non-pseudohyphal *S. cerevisiae* stains after 4 h in SLAD-1% (Figure 27). Under the same conditions, in *S. paradoxus* only FLO11, FLO8, RAS2, and PHD1 displayed significant differences in expression between pseudohyphal and Non-pseudohyphal strains (Figure 27). However, none of the genes had significant differences in expression between pseudohyphal and non-pseudohyphal *S. bayanus* strains (4 h, in SLAD-1%) (Figure 27).

Since the divergence between *S. cerevisiae* and *S. bayanus* transcription profiles could be a matter of difference in temporal regulation, I next expanded the examination of transcription and measured mRNA levels for a subset of pseudohyphal and non-pseudohyphal strains after 12 h in SLAD-1%. Neither species exhibited a difference in expression levels for the selected genes (FLO11, FLO8, GPA2, STE12, TEC1, TPK1, TPK2, TPK3) between pseudohyphal and non-pseudohyphal strains (Figure 28).

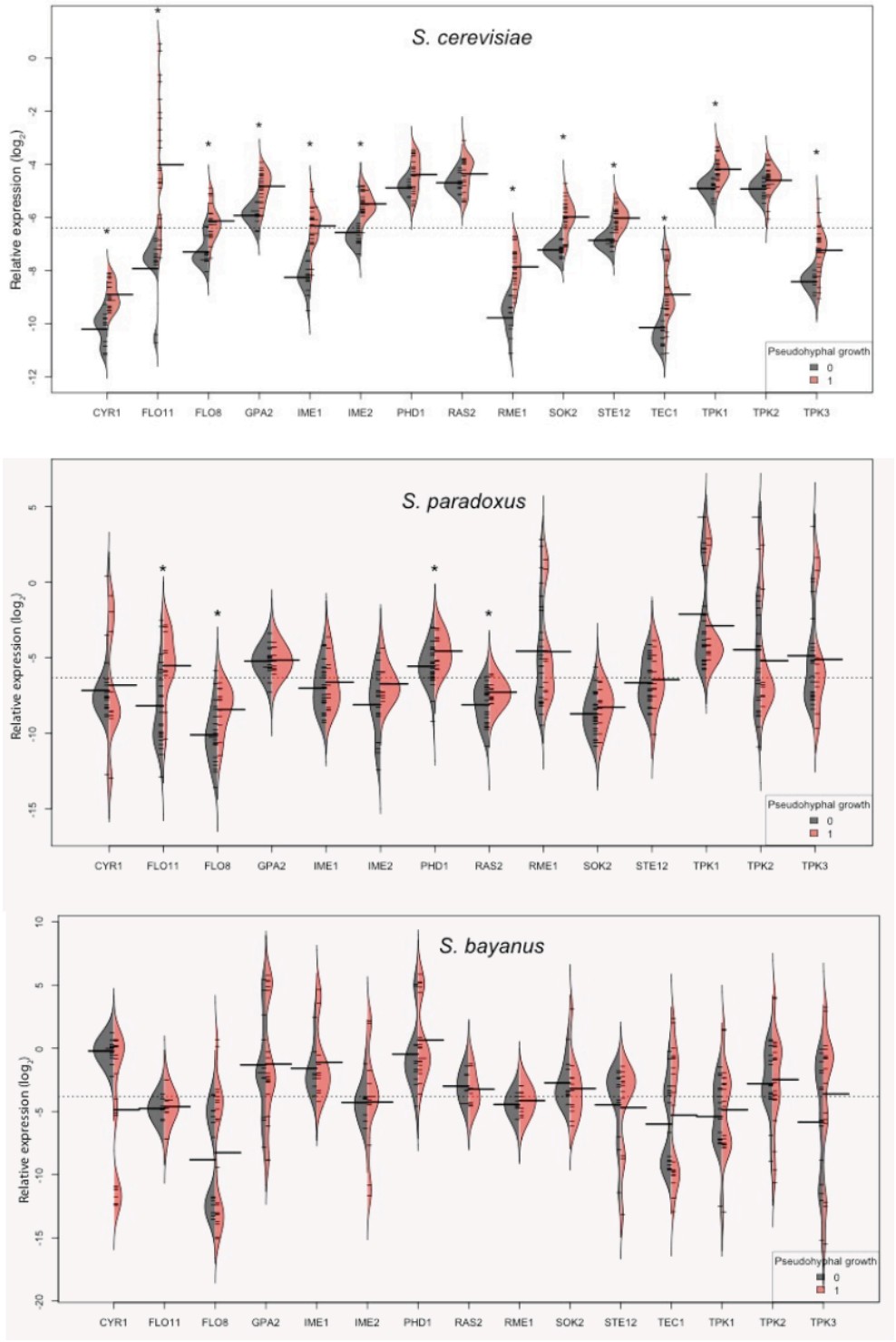

Figure 27 Differential transcriptional profiles and divergence
Joint histograms of expression levels for key genes in pseudohyphal and non-pseudohyphal *Saccharomyces* strains under nitrogen depletion, 4 h. Black: Non-pseudohyphal, Red: Pseudohyphal The black bars in the histograms indicate the mean. (*) marks the significant differences at ($p<0.05$) Expression levels normalized to ACT1

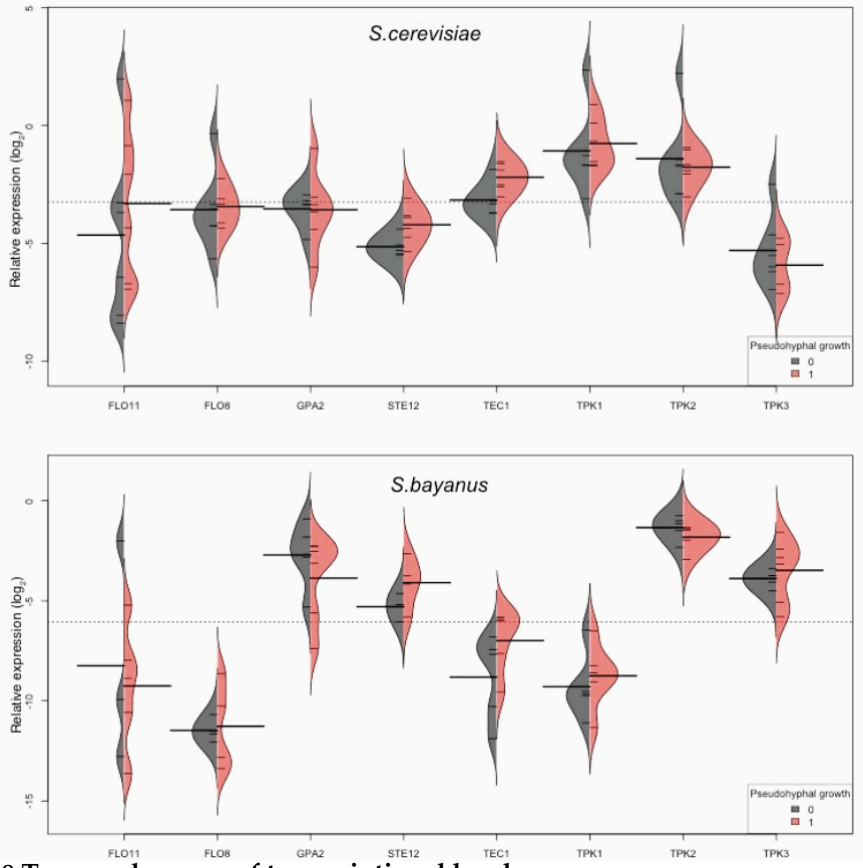

Figure 28 Temporal survey of transcriptional levels.
Expression levels in a subset of pseudohyphal and non-pseudohyphal strains for each species under nitrogen depletion, 12h.

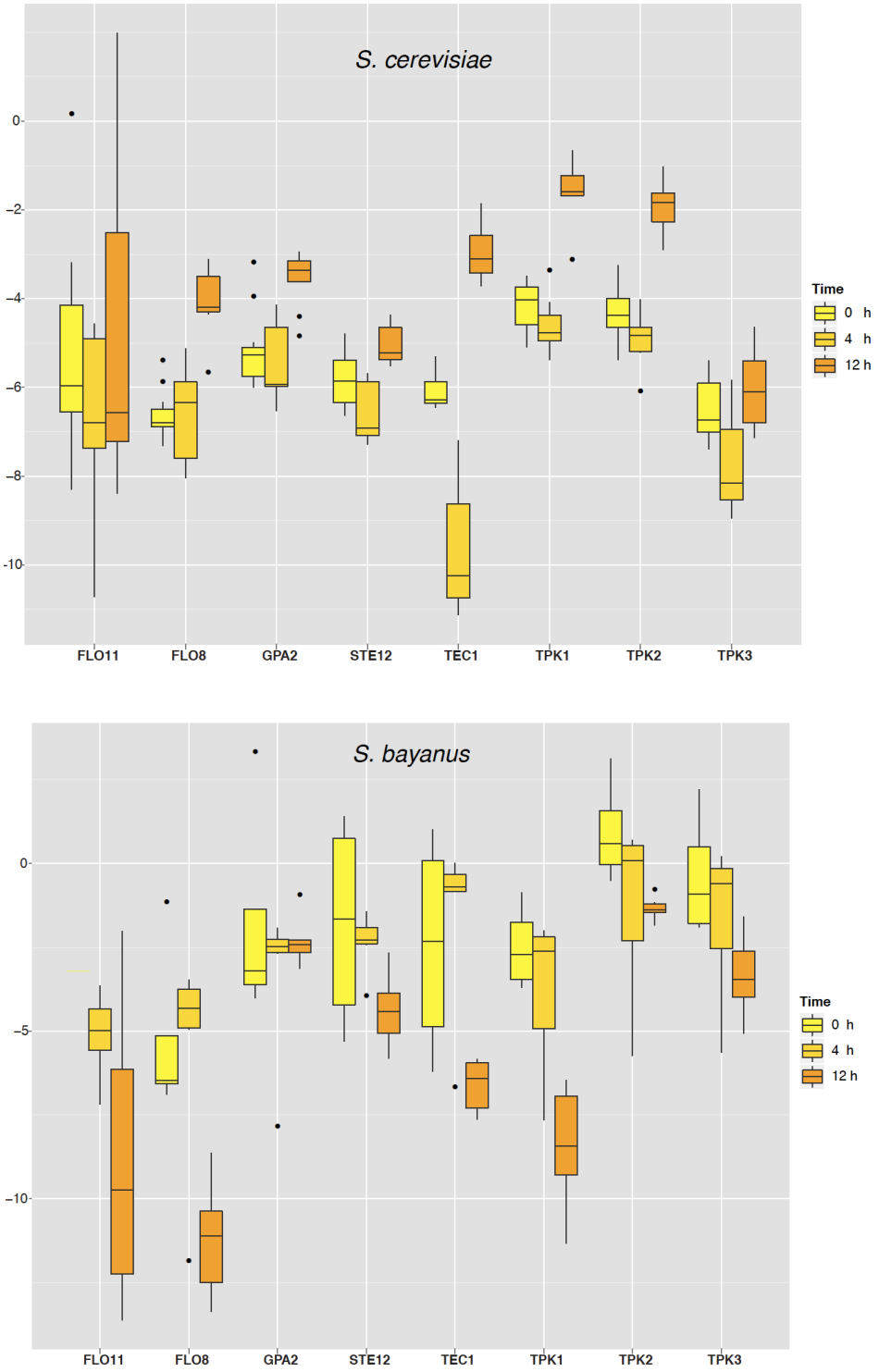

Figure 29 Gene expression trends

Box plots showing expression levels for 8 genes in a subset of *S. cerevisiae* and *S. bayanus* in nitrogen-deficient media

FLO11 Expression and pseudohyphal phenotypes are not correlated in *S. bayanus*

Since Flo11 is essential for pseudohyphal differentiation, transcription levels for FLO11 have been used as a quantitative proxy for the response in *S. cerevisiae*. I further investigated the divergence in regulation of pseudohyphal growth at genetic and transcriptional levels focusing on FLO11 expression between the two species. However, if FLO11 were a dispensable component in induction of pseudohyphal response then correlation between FLO11 expression and pseudohyphal growth would be absent in *S. bayanus*. Remarkably, the examination of transcript levels for FLO11 in *S. bayanus* null mutants under nitrogen-limiting conditions, indeed revealed a lack of detectable correlation between FLO11 transcription dynamics and pseudohyphal differentiation.

FLO11 expression appeared to follow different dynamics between *S. cerevisiae* and *S. bayanus* wild type strains in SLAD-1%. In *S. cerevisiae*, FLO11 transcript levels showed a gradual increase after an initial decline recorded at 2 h, and was about twofold higher at 24h ($p < 0.05$). Conversely, *S. bayanus* displayed more than a twofold increase at 2hr followed by a sharp decline with only two-thirds of initial expression levels at 24 h ($p < 0.05$) (Figure 30). Moreover, a temporal gene expression survey for cAMP-PKA elements also seems to exhibit a different trend in *S. cerevisiae* and *S. bayanus*(Figure 29). It would be interesting to further interrogate the apparent divergence in FLO11 temporal expression levels in *S. bayanus* relative to *S. cerevisiae* for a longer time-course and a larger set of strains.

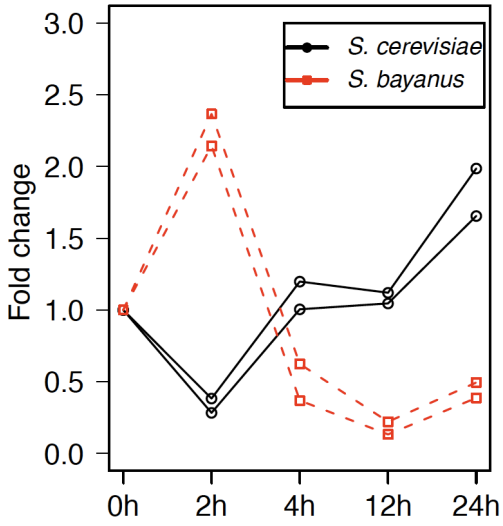

Figure 30 FLO11 expression over a time course
The mRNA levels for FLO11 appears different in *S. cerevisiae* (Σ 1278b) and *S. bayanus* (PMY640) in nitrogen deficient media, as measured by qPCR.

The levels of FLO11 mRNA in *S. bayanus* MAPK mutants, overall, had similar temporal patterns to the wild type; an incline followed by a sharp decline. Unlike, *ste7Δ*, *ste12Δ* and *tec1Δ* strains, which had similar expression levels to the wild type over different time points, the *dig1Δ* mutant showed a fourfold hike in FLO11 expression at 2 h in line with observed increase in pseudohyphal growth and repressor activity reported in *S. cerevisiae* ($p<0.05$) (Figure 31). Expression levels were significantly lower, relative to wild type, in *ste7Δ*, *ste12Δ* and *tec1Δ* strains of *S. cerevisiae* (Figure 31).

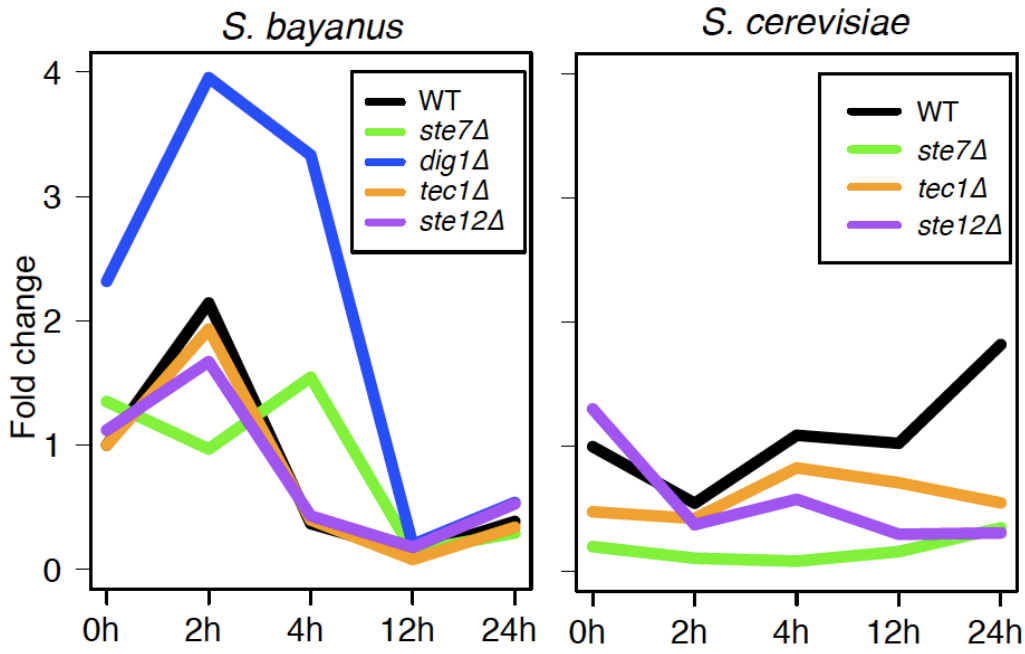

Figure 31 Similar phenotypes, different expression profiles
The expression levels for FLO11 are different upon MAPK inhibition in *S. bayanus* and *S. cerevisiae* under limiting nitrogen, as measured by qPCR over a 2-24 h time course. The disruption of MAPK eliminates pseudohyphal response in both species.

FLO11 mRNA levels and pseudohyphal phenotype did not show a correlation in *S. bayanus* strains with defective cAMP –PKA signaling (Figure 32a). Temporal dynamics for FLO11 transcript levels were also a similar in these mutants when compared to the wild type strain. However, there were exceptions to the observed lack of correlation as in the case of *tpk3Δ*, *sfl1Δ*, and *phd1Δ*. Tpk3 and Sfl1 are strong suppressors of FLO11 expression in *S. cerevisiae*, and *S. bayanus* strains defective in these loci showed 70- and 90- fold higher FLO11 mRNA levels, respectively (Figure 32b). Also, unlike the rest, the *phd1Δ* strain that showed a complete loss of pseudohyphal growth had about half of the initial mRNA levels at all time points ($p < 0.05$) (Figure 32a). In contrast to *S. bayanus*, as predicted, FLO11 transcript levels corresponded to observed pseudohyphal response in the corresponding *S. cerevisiae* mutants (Figure 32a).

Mutations for direct (*tpk1Δ*, *tpk3Δ*, *sfl1Δ*) and indirect inhibitors (*ira2Δ*, *pde1Δ*) of the response had very high FLO11 transcript levels (Figure 32b).

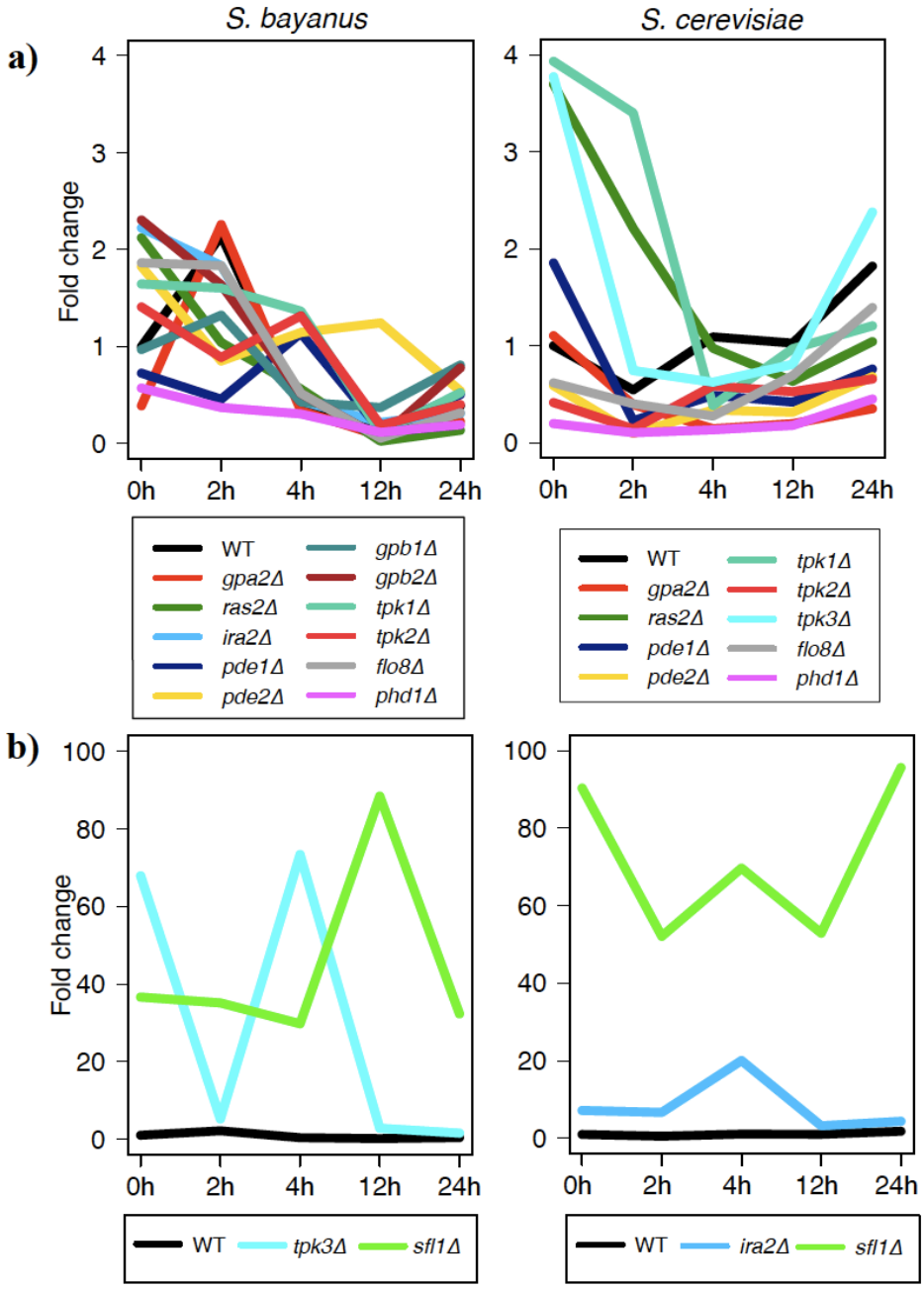

Figure 32 FLO11 expression and cAMP-PKA signaling
a) Defects in the cAMP-PKA signaling do not correlate between FLO11 expression and pseudohyphal development in *S. bayanus*. However, for *S. cerevisiae* the mRNA levels are consistent with the response and with the literature for the corresponding mutants **b)** Tpk3 and Sfl1, known inhibitors of pseudohyphal differentiation, exert similar effects on FLO11 expression and pseudohyphal growth in both species. Deletion of Ira2, a negative regulator of cAMP level and hence the response, exhibits increased FLO11 trancription in ***S. cerevisiae.***

Since *S. cerevisiae* and *S. bayanus* have adapted to different ecological niches and have different sensitivity to nutrient-stress I scored pseudohyphal response under different combinations of nitrogen and carbon levels. *S. cerevisiae* had stronger response to various levels of carbon and nitrogen levels when compared to the response on SLAD-1%. On the other hand, *S. bayanus* showed similar pseudohyphal growth under all conditions tested (Figure 33). Since PKA derepresses general stress-response mechanism under pseudohyphal conditions I also examined effects of known stress-response regulators in *S. bayanus*. I deleted SCH9, RIM15 and MSN2 that play an important role in mediating stress-response in *S. cerevisiae*. PKA and Sch9 exert a negative regulation on Rim15 independently, and this inhibition is relieved upon nutrient limitation, leading to activation of Msn2 by Rim15 for induction of stress-responsive gene expression[32,107]. According to the interactions in *S. cerevisiae*, deletion of SCH9 results in a strong pseudohyphal phenotype whereas deletion of RIM15 and MSN2 eliminate pseudohyphal differentiation. Strikingly, however, null mutations of these loci displayed exact reverse phenotypes in *S. bayanus*: *sch9Δ* had a complete loss of pseudohyphal response while *rim15Δ* and *msn2Δ* had comparable pseudohyphal response to the wild type (Figure 34).

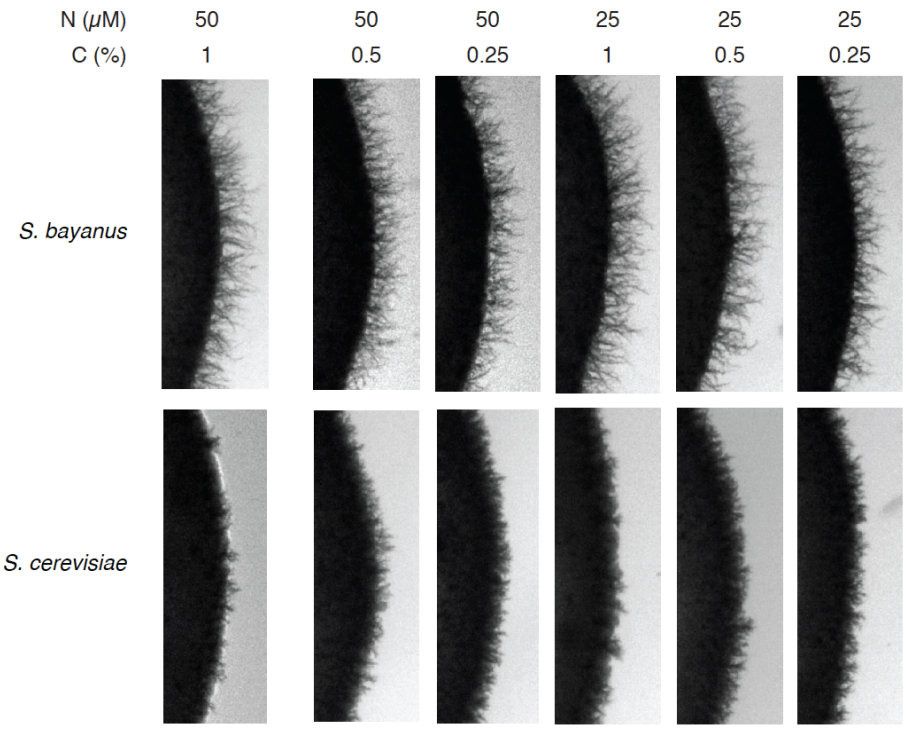

Figure 33 Divergence in stress sensitivity
S. cerevisiae and *S. bayanus* display different sensitivity to different combinations of Carbon and Nitrogen concentrations as surveyed by six strains for each species.

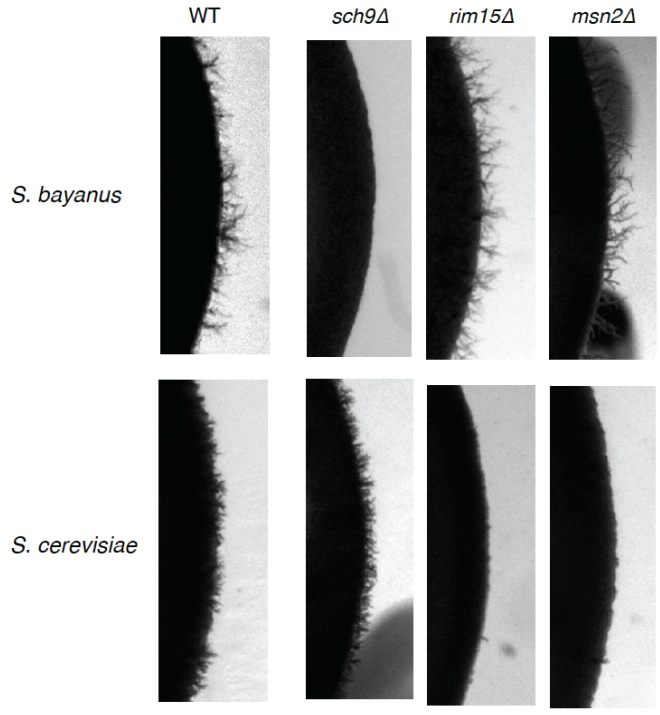

Figure 34 Divergence in stress-response transcription factors
Deletion of stress-response elements shows opposite effects on pseudohyphal response

3.3 Discussion

Understanding functional interactions and evolution of regulatory networks is an important step for interpreting their roles in numerous cellular processes and responses under diverse conditions. Comparative analysis of cAMP-PKA signaling in *Saccharomyces* highlights that changes in interactions and connections between key components can lead to extensive differences in regulation and expression of phenotypes important for survival. In this work I show that the cAMP-PKA pathway has an alternative regulatory mode in coordination of pseudohyphal growth in closely related *S. cerevisiae* and *S. bayanus*. The cAMP-PKA pathway is a major regulator of nutrient-induced pseudohyphal growth in *S. cerevisiae.* The critical role and elements of this signaling pathway coordinating pseudohyphal differentiation has been studied extensively in *S. cerevisiae* as this form of development is also related to morphogenetic switch associated with virulence of human pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* under nutrient limitation [36,44,53,54].

Earlier work has shown that nutrient-induced pseudohyphal growth and sporulation responses exhibit a strong phenotypic trade-off in *S. cerevisiae* (Figure 6) [91]Examination of closely related *Saccharomyces* species, however, reveals that the inverse correlation between pseudohyphal growth and sporulation is very weak in *S. paradoxus* and completely absent in *S. bayanus* (Figure 14). This could be a result of the different physiologies and stress tolerance shaped by the species' preferred ecological niches[66].

Comparison of intracellular cAMP levels in the three species studied here underscored the importance of this secondary messenger for induction of pseudohyphal growth. Complementary to previous works showing cAMP promotes pseudohyphal growth in yeast[36], intracellular cAMP levels mirrored propensity for the response in *S. cerevisiae* and *S. paradoxus* but not in *S. bayanus* (Figure 15). First explanation comes to mind for the observed pseudohyphal efficiency in *S. bayanus* is that a low quantity of cAMP is sufficient to induce pseudohyphal growth in this species. Yet, results from the follow up experiments contradict the existing model for the role of cAMP signaling in *Saccharomyces*, and implies significant rewiring in the pseudohyphal differentiation network. While exogenous cAMP exaggerates pseudohyphal response in *S. cerevisiae* and *S. paradoxus*, presence of cAMP in the media completely inhibits pseudohyphal switch in *S. bayanus* (Figures 16- 17). The pharmacological agents that modulate cAMP levels also supports the impact of exogenous cAMP has on the response in *S. bayanus* (Figure 18). Antagonistic regulation of filamentous response by cAMP-PKA signaling has been also observed in *Yarrowia lypolytica* and *Ustilago maydis* but these species carry out entirely distinct physiologies and occur in quite divergent habitats than *Saccharomyces* [50,54,55]. So, contrasting effects of exogenous cAMP treatment on closely related *Saccharomyces* species is remarkably surprising.

MAPK signaling is another key signaling pathway that regulates pseudohyphal growth. Unlike cAMP-PKA signaling, MAPK cascade is a stress-activated pathway and mediates stress-induced responses from yeast to man [114,115]. Thus, disruption of MAPK cascade in *S. bayanus* confirms that the impact of kinases (Ste7 and Dig1) and

downstream transcription factors (Ste12 and Tec1) for critical pseudohyphal growth is conserved and essential in related *Saccharomyces* species (Figure 19).

Components of the cAMP-PKA signaling in *S. cerevisiae* and their homologs in *C. albicans* and *C. neoformans* are required and have similar functions in filamentous differentiation [47,52,54]. Loss of function mutations in several upstream elements of the cAMP-PKA signaling show that the activity of the pathway is also required and important for cellular differentiation in *S. bayanus*. Specifically, pseudohyphal phenotypes of knock-out mutants for genes including GPA2, TPK1, TPK2, TPK3 and RAS2 indicate the critical role of cAMP-PKA signaling in *Saccharomyces*. Gpa2 impacts pseudohyphal response via its positive effects on Cyr1 and via its negative effects on Krh(Gpb) proteins that stabilize inhibitory effects of the regulatory subunit of PKA (Figure 20-21) [116,117]. Moreover, consistent results on the contrasting roles of PKA catalytic subunits on pseudohyphal regulation are also in accord with the reported activities of these elements in *Saccharomyces* and human fungal pathogens *C. albicans* and *C. neoformans* indicating that the important role of core components have been maintained [37,42,115].

Interestingly, however, Ras2 deletion only moderately impacted pseudohyphal growth despite its function as an activator of both cAMP-PKA and MAPK signaling and a total loss of pseudohyphal response in the corresponding mutant in *S. cerevisiae* [35]. An explanation for this surprising observation could be a different post-translational modification of the protein. A number of putative phosphorylation sites have been described on Ras2, however data indicate that only one of these sites (S^{214}) is

preferentially phosphorylated and critical [116,117]. Activated Ras2 protein is hypophosphorylated, and phosphorylation of S^{214} has been shown to negatively regulate Ras2 function. Besides, S214A point mutation manifests constitutively active Ras2 phenotypes, including heat sensitivity and elevated cAMP levels [117].

Amino acid alignment of Ras2 for a number of *S. bayanus* strains including the published sequences showed that the PKA phosphorylation motif -RKxS- including (S^{214}) has been degraded. This is consistent with, as discussed below, inhibition of pseudohyphal response by elevated cAMP-PKA signaling in *S. bayanus*. Another possible explanation for this result is that regulation of PKA could also happen in a cAMP-independent manner through Krh(Gpb) proteins. In fact, moderate increase in magnitude of pseudohyphal response upon loss of Krh proteins supports contribution of this branch to the response (Figure 12, 20). Furthermore, the slight defect in pseudohyphal growth after deletion of Ras2 homolog Ras1, which in *C. albicans* and *C. neoformas* is also the key activator for both cAMP-PKA and MAPK signaling pathways[45,115] show that Ras1 also has a part in pseudohyphal growth in *S. bayanus* (Figure 20). Together, individual disruption of upstream components in the pseudohyphal network suggests that three different branches of upstream interactions could carry out activation of PKA and pseudohyphal growth collectively. This also emphasizes that the roles for the majority of upstream components have been maintained between *S. cerevisiae* and *S. bayanus.*

The feedback mechanism controlling cAMP levels in *S. bayanus* shows a stark contrast in regulation of pseudohyphal response. GTPase-activating proteins and

phosphodiesterases control intracellular-cAMP levels[109,118–120]. As demonstrated in *S. cerevisiae*, functional defects in Ira2 and Pde1 lead to elevated cAMP concentrations that exaggerate pseudohyphal growth[34]. Corresponding mutations in *S. bayanus*, however, strikingly abolished pseudohyphal growth in *S. bayanus* supporting results from the experiments where cAMP levels were modulated externally (Figures 22). This, again, underscores that cAMP secondary messenger is an important mediator in regulation of filamentous differentiation, yet in an alternative fashion. Support for this alternative effect of elevated cAMP levels transmitted through PKA in *S. bayanus* comes from the *bcy1Δ* mutant that exhibits hyperactive PKA. Like in *S. cerevisiae*, hyperactivation of PKA also led to thermo-sensitivity and reduced growth rates in *S. bayanus*. However, failure of the mutant to differentiate into pseudohyphal form revealed that not only elevated cAMP levels but also functions that pertain to increased cAMP levels such as PKA activation mediate inhibition of pseudohyphal growth in *S. bayanus* (Figure 23). Thus, antagonistic effects of the cAMP-PKA signaling in *S. bayanus* are mediated through the negative feedback controlling cAMP levels and its major effector PKA.

Observed data also suggest that while core upstream operations and interactions of the cAMP-PKA have been maintained the divergence in regulation of pseudohyphal switch is due to altered interactions between downstream effectors. This is supported by induction of pseudohyphal response in the *flo8Δ* mutant despite, as in *S. cerevisiae*, deletion of SFL1 and PHD1 inhibited the response (Figure 24). Flo8, Sfl1 and Phd1 are key transcription factors downstream of the cAMP-PKA signaling regulating

pseudohyphal response via their effects on FLO11 expression[14,36]. PKA subunit Tpk2 promotes pseudohyphal growth by negatively regulating Sfl1 transcription repressor and by positively effecting transcription activators Flo8 and Phd1. Beside directly interacting with and upregulating FLO11, Flo8 also indirectly effects FLO11 expression by promoting PHD1 expression. Moreover, interaction of Flo8 with FLO11 promoter renders the upstream region available for trans-acting elements [36,124]. Besides, inhibition of pseudohyphal growth upon deletion of Flo8 and Phd1 not only under nitrogen limitation but also in presence of aromatic alcohols in *S. cerevisiae* highlights importance of these transcriptional factors for pseudohyphal growth [122,123]. Hence, ability of *S. bayanus* to undergo pseudohyphal switch and invade agar with a deleted FLO8 strongly supports alternative functional interactions and/ or additional regulatory connections participating in regulation of pseudohyphal growth.

Another compelling evidence that points to an altered set of downstream interactions is the induction of late-onset pseudohyphal growth in *flo11Δ* mutant (Figure 25). This result not only supports alternative interactions but also indicates additional downstream targets playing a role in pseudohyphal response. Wealth of data has implied FLO11 as an exclusive downstream target indispensable for nutrient-induced pseudohyphal growth. Although epigenetic control mechanisms and variations in internal tandem repeat numbers impinging on FLO11 transcription have been noted to influence pseudohyphal propensity in *S. cerevisiae*, null mutation of FLO11 has always been shown to eliminate nutrient-induced pseudohyphal growth[34,39]. At this point, it is important, however, to mention that FLO11 has been reported to be dispensable for

pseudohyphal differentiation triggered in presence of aromatic alcohols [121,124]. Additionally, it has been shown that loss of pseudohyphal growth upon FLO11 deletion can be compensated by overexpression of another FLO gene-family member, FLO10 [125]. FLO10 encodes a cell surface glycoprotein that is also under regulation of abovementioned transcription factors downstream of cAMP-PKA and MAPK signaling that control FLO11 and pseudohyphal growth [126,127]**.** Together with this information, then, the alternative functional interactions hypothesis predicts that beside FLO11, FLO10 is a highly likely downstream target that underlies pseudohyphal growth in *S. bayanus*. Moreover, as observed for *S. cerevisiae,* increased FLO10 mRNA abundance in absence of IRA2 thereby upon elevated cAMP levels denotes that contributions of this adhesin could be on par with FLO11 input in *S. bayanus* [128].

The unpredicted moderate increase in pseudohyphal growth upon pharmacological inhibition of adenylate cyclase (Cyr1) might provide some insight into possible interactions regulating cAMP-PKA mediated inhibition of pseudohyphal growth in *S. bayanus* (Figure 26). Blocking adenylate cyclase function prevents pseudohyphal growth with reduced internal cAMP level, which under these circumstances is insufficient to trigger critical interactions in *S. cerevisiae* and *S. paradoxus*. Conversely, increased pseudohyphal response upon Cyr1 inhibition and pseudohyphal repression in the *bcy1Δ* mutant suggest that inhibitory interactions are dominant when cAMP-PKA signaling is over-activated. Stronger pseudohyphal response in all but the *phd1Δ* mutant upon addition of adenylate cyclase inhibitors also supports this hypothesis (Figure 26). Meanwhile, a possible explanation for the *phd1Δ*

case could be that this locus is likely to be under more stringent conservation because Phd1 has been described as a master regulator of pseudohyphal differentiation in *S. cerevisiae*. This critical role of Phd1 is, probably, also the case in *S. bayanus* since Phd1 has been demonstrated to be a highly connected transcription factor and a predominant participant in downstream interactions affecting pseudohyphal growth [101,129,130]. This result also implies that Phd1 probably stands at a major intersection that mediates antagonistic effects of cAMP-PKA signaling on pseudohyphal growth in *S. bayanus*.

Distinct transcriptional profiles provide further evidence for alternative downstream targets and dynamic interactions coordinating the negative role of cAMP-PKA signaling in *S. bayanus.* The contrast manifested in expression levels obtained for the set of developmental genes in pseudohyphal and non-pseudohyphal *Saccharomyces* strains mirrors divergent signaling and functional interactions underlying developmental processes in the lineage. The apparent difference in expression dynamics for FLO11 is agrees with the phenotypic, genetic and signaling data, and further supports the idea that additional players and altered connections are contributing to pseudohyphal growth in *S. bayanus*. The observed early increase in FLO11 mRNA levels may indicate that *S. bayanus* turn on activation upon transfer into nitrogen limiting media while the decrease in *S. cerevisiae* suggests that there is an existing pool of FLO11 transcripts that are immediately translated upon depletion of nitrogen (Figure 30). Also, unique dynamics and expression levels for FLO11 in the MAPK mutants of *S. cerevisiae* and *S. bayanus* further emphasize presence of other players (Figure 31). Although inhibition of pseudohyphal growth is consistent with disruption of MAPK

signaling in both species, comparable expression dynamics in the wild type and mutants strains strongly suggest that FLO11 is dispensable for pseudohyphal growth in *S. bayanus*.

Lack of correlation between FLO11 expression dynamics and pseudohyphal response in *S. bayanus* strains with defective cAMP-PKA signaling, again, shows that cAMP-PKA signaling also targets additional adhesins for pseudohyphal differentiation (Figure 32). Unlike for *S. bayanus*, the abundance of FLO11 mRNA is consistent with pseudohyphal response and with previous studies for *S. cerevisiae* strains defective in cAMP-PKA signaling[117]. This underscores importance of Flo11 for pseudohyphal growth in *S. cerevisiae* (Figure 32).

Nevertheless, although the transcription levels look random in *S. bayanus*, mRNA abundance for FLO11 should be noticed for two of the mutants. First, the *sfl1Δ* strain has the highest increase in expression levels and pseudohyphal response confirming that Sfl1 is a strong inhibitor of pseudohyphal growth both in *S. cerevisiae* and *S. bayanus* (Figure 32b). Second, the substantial hike observed for FLO11 transcript abundance associated with the pseudohyphal response in the *tpk3Δ* mutant is consistent with the inhibitory role of Tpk3 in regulation of pseudohyphal growth. Moreover, the significant 60-fold increase and oscillatory behavior for FLO11 transcript levels in *S. bayanus* indicate that Tpk3 exerts a much more effective inhibition on pseudohyphal growth in this species. In contrast, Ira2 seem to be the second strongest inhibitor (at least in the set of genes examined) for *S. cerevisiae* and has about ten times the effect on FLO11 expression compared to Tpk3 in *S. cerevisiae* (Figure 32). Therefore, the distinct

FLO11 expression profiles for *S. cereveisiae* and *S. bayanus* hints at altered interactions of inhibitors are pivotal in cAMP-PKA regulation of pseudohyphal growth negatively.

With the data in hand, one possible mechanism that cAMP-PKA signaling could mediate the antagonistic regulation of pseudohyphal growth in *S. bayanus* would be through differential activation of PKA and PKA-regulated transcriptional elements under varying cAMP levels. Higher binding affinities between the regulatory subunit (Bcy1) and the inhibitory catalytic subunits (Tpk1 and Tpk3) with more potent suppressor effects on downstream transcription factors require higher concentrations of cAMP levels for activation of Tpk1 and Tpk3 that strongly inhibits pseudohyphal growth. Accordingly, under pseudohyphal inducing conditions when cAMP levels are low PKA remains in its inactive holoenzyme form. Following a moderate increase in cAMP concentrations only the activating subunit (Tpk2) is released and in turn pseudohyphal growth is triggered. At the same time Tpk1 and Tpk3 are still bound by Bcy1 due to insufficient increase in cAMP levels for their activation (Figure 35).

As discussed earlier, the catalytic subunits (Tpk1, Tpk2, Tpk3) are known to target overlapping set of proteins that exert positive and/or negative influence on cellular differentiation. Actually, although the inhibitory effect of Tpk3 on pseudohyphal growth is undoubted, how it prevents pseudohyphal response is still unknown [12,37]. Unlike Tpk1 and Tpk2, Tpk3 has not been studied as extensively probably due its hardly

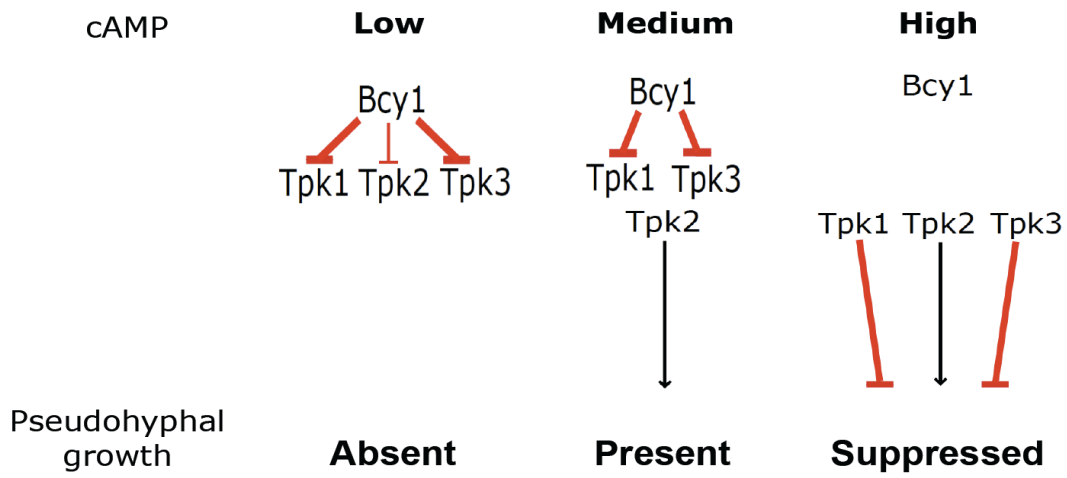

Figure 35 A model for cAMP-PKA signaling in *S. bayanus*
Differential affinities and interactions of PKA subunits are critical in the divergent role of the cAMP-PKA signaling in development. Moderate levels of cAMP dissociate the activating subunit and triggers pseudohyphal response whereas at higher levels cAMP activates the inhibitory PKA subunits with stronger inhibitory impact and suppress pseudohyphal growth.

detectable levels of mRNA expression and protein activity [131,132]. Actually, differential mRNA levels for the regulatory subunits are also detected in the expression data presented here here. Tpk3 mRNA expression is one-third of the levels for Tpk1 and Tpk2, and one-fourth of the Tpk3 expression in *S. bayanus*. Moreover, existing work on the PKA subunits suggests that beside indiscernible expression and activation levels, Tpk3 has the lowest affinity for the regulatory subunit (Bcy1) in *S. cerevisiae*[133]. In addition to differential activation of the Tpks, alternative interactions on the promoter might also contribute to combinatorial transcriptional regulation of adhesins in *S. bayanus*. In fact, presence of polyglutamine domains in different lengths at N-terminal of the catalytic subunits as wells as of key proteins, such as Sfl1, Mga1, Mss11, point to different protein/DNA and protein/protein interactions.

The general-stress response mechanism seems rewired in *S. bayanus*. Strong pseudohyphal response of *S. bayanus* at different combinations of Carbon and Nitrogen concentrations in contrast to varying strength of pseudohyphal response of *S. cerevisiae* underlines divergent stress sensitivities between the species (Figure 34). Interestingly, however, the surprising result that disruption of key transmitters in the general-stress response mechanism caused the exact opposite effects on pseudohyphal growth in *S. cerevisiae* and *S. bayanus* suggests that this mechanism may have also undergone rewiring. Consistently, loss of pseudohyphal response upon deletion of Sch9 implies that this nitrogen responsive kinase is important for pseudohyphal regulation in *S. bayanus* (Figure 34). Moreover, this result suggests alternative interactions between cAMP-PKA and TOR signaling, which targets Sch9 directly[30,134,135].

Collectively, comparative analysis of cAMP-PKA signaling in this study indicate that reorganization of interactions and connections between existing components underlies strategic differences in regulation and expression of phenotypes important for cellular functions and survival. I expect that that these distinct interactions are reflective of environmental conditions that cells experience in their adapted niches.

4. Oscillation and variation of cAMP levels in *Saccharomyces*

4.1 Introduction

The cyclic AMP-Protein Kinase A (PKA) signaling propagates changing nutritional conditions captured by sensing mechanisms in the plasma membrane to the nucleus for activation and/or repression of multitude of genes and proteins to promote growth and development. In response to nutrient stimuli, activation of PKA in the eukaryotic model system, *Saccharomyces cerevisiae*, is directed by intracellular cAMP concentrations, an important second messenger [11]. Levels of cAMP are controlled by a feedback mechanism (Figure 36), and in derepressed yeast cells show a significant transient increase in response to fermentable carbon sources, such as glucose [109]. Unlike in lab conditions, nutrients are neither easily accessible nor available for long periods for yeast cells in nature. Hence, the dramatic rapid increase in the cAMP levels upon transition from starvation to nutrient rich conditions could provide an advantageous trait for efficient proliferation in their natural environment.

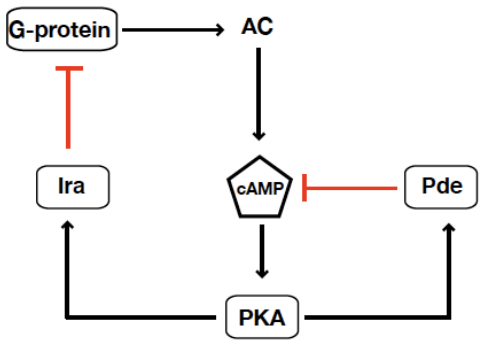

Figure 36 Negative feedback regulation of cAMP levels
Antagonistic activities of adenylate cyclase (AC) and phosphodiesterases (Pde) balances cAMP levels in a cell.

An increase in internal cAMP levels upon glucose induction activates cAMP-dependent PKA. Binding of cAMP molecules to the regulatory subunit (Bcy1) is ensued by a conformational change releasing and activating the catalytic subunits (Tpk1, Tpk2, and Tpk3) [10,11,93]. Activation of PKA triggers a negative feedback loop that mediates the rate of synthesis and hydrolysis of cAMP carried out by adenylate cyclase (AC) and phosphodiesterases (Pde1 and Pde2), repectively. In yeast, PKA activates Pde1 (the low-affinity Pde) and Pde2 (the high-affinity Pde) by phosphorylation, and by monitoring the intracellular cAMP levels Pde1 and Pde2 regulate the activity of PKA [11,109]. In this mechanism, Pde2 degrades cAMP to maintain a basal level of the molecule, and Pde1 regulates the rapid increase in levels of cAMP required for activation of PKA[109]. Besides, in this feedback loop, PKA also inhibits AC activity probably by phosphorylating Ira1 and Ira2, GTPase activating proteins of Ras2 [120]. Consequently, progression of many signal transduction cascades is controlled by this tightly regulated negative feedback inhibition of cAMP-mediated PKA activity.

cAMP-PKA signaling regulates numerous signaling pathways important for survival and development. Given the variation observed for nutrient-induced developmental responses within and between *Saccharomyces* species, in this study, I attempt to investigate variation in short-term dynamics of cAMP levels upon glucose induction. Intra- and inter-*Saccharomyces* variations observed in dynamics, amplitude and duration of the transient change in cAMP levels in addition to variation in amino acid and promoter sequence for loci involved in the negative feedback loop present the

cAMP signal as a useful molecular trait to employ for investigating how variation in genotypes contribute to variations in phenotypes.

4.2 Materials and Methods

Strains, Media and cAMP Assay

Diploid *Saccharomyces* strains were used in this study (Table 8). Cells from independent cultures were prepared following a protocol adapted from Paiardi et.al., 2007 [136]. About 2x 10^9 cells/ml were grown in rich media (1% yeast extract, 2%peptone and 2% glucose) at optimal temperatures (room temperature for *S. bayanus* and 30 °C for the rest) and transferred to synthetic complete (SC) media with 0.1% glucose and 3% gycerol for overnight incubation at 30 °C. Starving cells were collected, washed and incubated in 25mM MES buffer, pH6 (Boston Bio Products, Worcester, MA) for 30 minutes at optimal temperatures before glucose induction. Glucose added to 2ml of cell culture (~ 2x 10^8 cells/ml) to a final concentration of 100 mM.

After glucose induction cells were collected over an eight- to twelwe-minute time course in 15- second increments for the first minute and 30-second increments for the rest. 3.4 x 10^6 cells collected at each time point were fixed in 300 µl n-butanol-saturated 1M formic acid and frozen at -80°C. Cells then lysed by four freeze-thaw cycles before freeze-dried in Speed Vac concentrator (Savant Instruments, Farmingdale, NY). Cell extracts were used to determine cAMP concentration using cAMP Biotrak Enzymeimmunoassay kit (Amersham, GE Healthcare).

Table 8 List of strains (Chapter 4)

Species	Strain	Genotype	Reference
S. cerevisiae	S288c	WT	SGRP Collection
	W303	WT	SGRP Collection
	SK1	WT	SGRP Collection
	Σ1278b	WT	Drees, *et.al.*, 2005
	PMY 508	Σ1278b *tpk3Δ:: kanMX*	Drees, *et.al.*, 2005
	PMY 746	Σ1278b *pde1Δ:: kanMX*	Drees, *et.al.*, 2005
	PMY 750	Σ1278b *pde2Δ:: kanMX*	Drees, *et.al.*, 2005
	PMY 1013	S288c *tpk3Δ:: kanMX*	SGD deletion consortium
	PMY 1216	S288c *tpk3Δ:: kanMX*	SGD deletion consortium
S. paradoxus	YPS 138	WT	SGRP Collection
S. bayanus	CBS 380	WT	SGRP Collection

4.3 Results

cAMP levels oscillate in derepressed *S. cerevisiae* cells upon glucose induction

S288c, Σ1278b, W303 and SK1 were chosen for their importance and frequent use in research. S288c is the standard reference strain for yeast studies while Σ1278b is commonly used for studying pseudohyphal differentiation, and W303 and SK1 are mostly used for meiosis and sporulation research. Nucleotide divergence from S288c for W303, Σ1278b, and SK1 is estimated to be 0.08%, 0.24% and 0.36%, respectively[137]. Measurements of cAMP concentrations at high temporal resolution for commonly used diploid lab strains (S288c, W303, Σ1278b, and SK1) displayed a rapid significant increase of cAMP levels that reached lower steady-state concentrations with decaying oscillations (Figure 37). In fact, mathematical modeling of the pathway by Gonzales et. al. (2011) predicted this dampened oscillations approaching steady-state levels for *S. cerevisiae*, and the results I obtained provided experimental evidence for the model [138].

Each strain had different oscillatory dynamics and exhibited differences in amplitude, duration or frequency of the decaying oscillations during eight- to twelve-minutes time-series. These different oscillatory dynamics could be reflective of genetic divergence between the strains. The highest amplitude was observed for Σ1278b, which was about twice the magnitude of the other three strains. Also, Σ1278b together with W303 showed high frequency decaying oscillations. Conversely, S288c and SK1 displayed a delayed decay in oscillations. All four strains had their initial cAMP peak within 3 minutes, with W303 and SK1 showing the earliest peak at 30s and 60s, respectively (Figure 37).

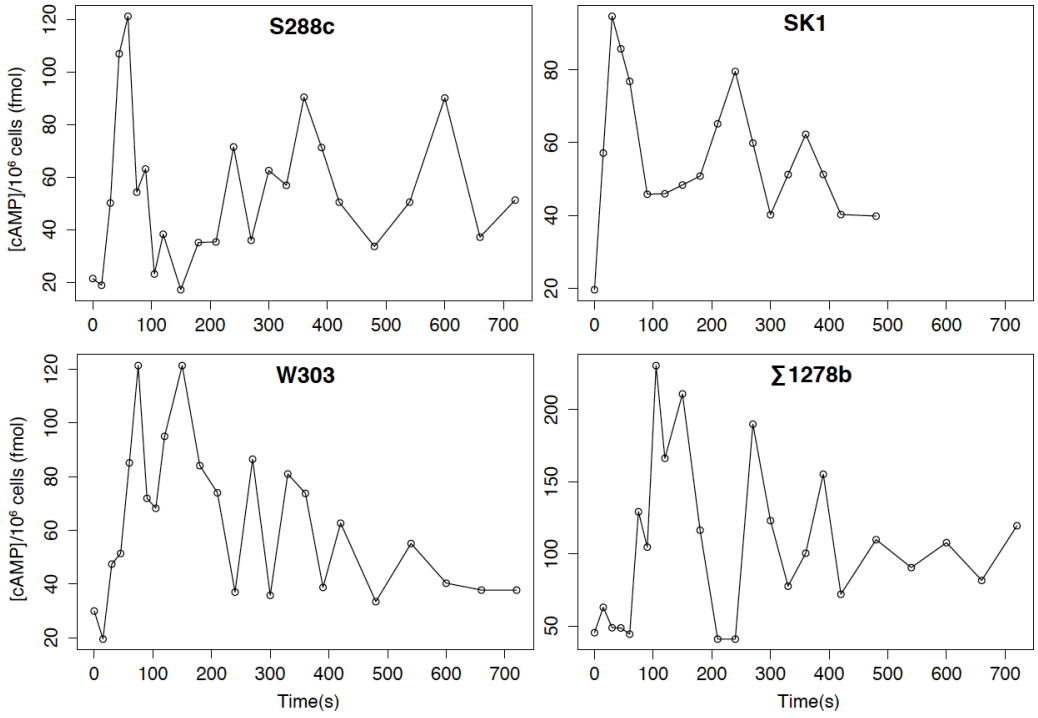

Figure 37 cAMP levels oscillate in *S. cerevisiae*
Intracellular cAMP levels oscillate in starved cells upon glucose replenishment. Strains exhibit distinct oscillatory patterns, which may underlie distinct developmental decisions. cAMP levels measured with ELISA.

cAMP oscillations are conserved in *Saccharomyces*

Since *Saccharomyces* species differ in their choice of preferred habitat I wanted to see whether these species would also show cAMP oscillations upon glucose replenishment. I measured cAMP levels in *S. paradoxus* and *S. bayanus* upon transition from starvation to rich media (20% glucose) (Figure 38). Consistent with results from *S. cerevisiae,* in *S. paradoxus* and *S. bayanus* cAMP levels also oscillated to steady-state concentrations after a considerable cAMP buildup. Nonetheless, *S. paradoxus* and *S. bayanus* showed an initial peak that was delayed and had a lower magnitude. Furthermore, while *S. paradoxus* exhibited comparable oscillation durations *S. bayanus* showed longer durations relative to *S. cerevisiae* (S288c) **(Figure 38)**.

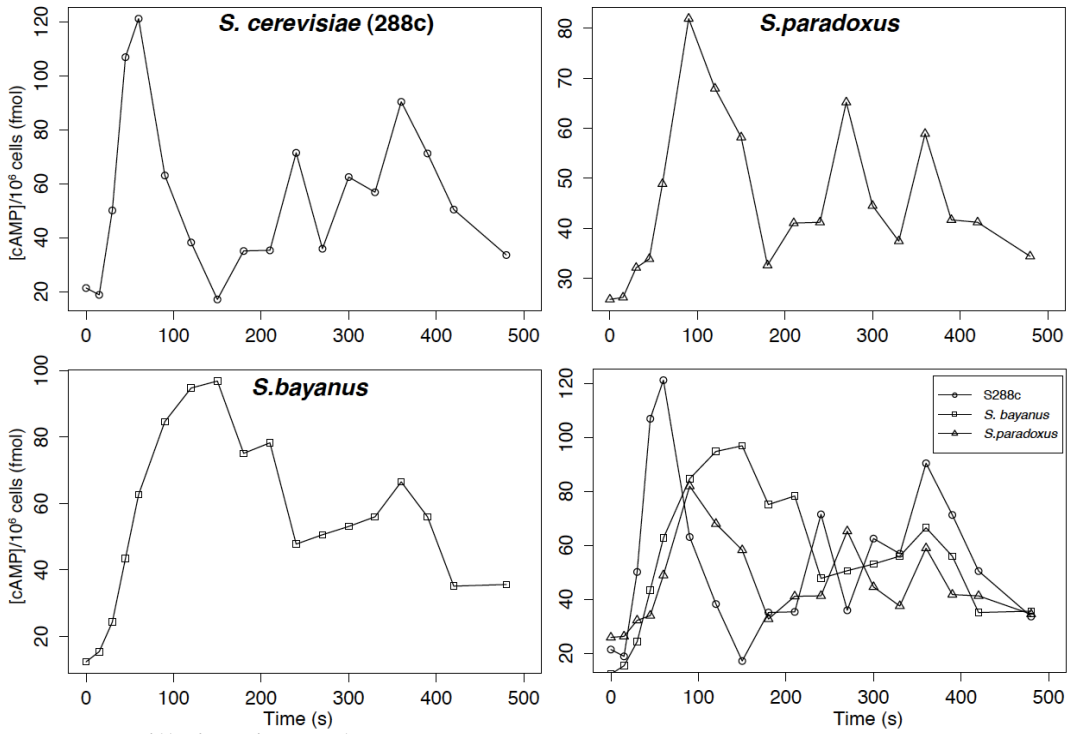

Figure 38 cAMP oscillations in *Saccharomyces*
Different *Saccharomyces* species display different patterns of oscillations as obtained by ELISA measurements.

Differential PKA activities may underlie variation in cAMP oscillations

SNPs present at coding and upstream regions of loci involved in the negative feedback inhibition of the cAMP-PKA signaling could contribute to inter/intra species variation observed in short-term cAMP dynamics. Since activation of PKA is a critical trigger for the feedback inhibition I first asked how mutations of the catalytic subunits affect the oscillations. It has been shown that each catalytic subunits (Tpk) can trigger the transient change in cAMP levels by itself yet with different intensities [118]

So, I predicted that deletion of a PKA catalytic subunit would cause a delay in activation of Pde proteins and thereby cAMP accumulates at different high concentrations for each Tpk mutant upon glucose induction. As expected, the S288c *tpk1Δ* mutant showed about 3.5 times the initial increase with a 30-second delay in the cAMP levels upon glucose replenishment relative to the wild-type strain. In this mutant, after the first peak, cAMP levels dropped immediately and showed oscillations at much lower concentrations, albeit higher compared to the wild-type strain (Figure 38). Similarly, the S288c *tpk3Δ* mutant exhibited about 2.5 times more cAMP accumulation (at exactly the same time point) and reached a steady state at comparable levels relative to the wild-type with no detectable oscillations (Figure 38).

Since Σ1278b exhibits a significantly different oscillation dynamics I also assayed cAMP levels in the Σ1278b *tpk3Δ* mutant strain. Tpk3 seemed to be a good candidate to start with because Σ1278b also has a non-synonymous SNP at catalytic domain of Tpk3 and as previous studies have described activation regulation of this subunit is different

than Tpk1 and Tpk2 [118,131]. Surprisingly, however, unlike in S288c, deletion of Tpk3 in Σ1278b caused cAMP levels to buildup sooner to only about 2/3 of the wild-type strain and showed delayed decay in oscillations (Figure 39).

4.3 Discussion

Fluctuations in cAMP levels are critical triggers for modulation of cellular growth and differentiation and hence tightly regulated [139–143]. The coordinative activities of AC and PDEs control concentrations of cAMP in response to a wide range of external stimuli [5]. In this study, I examine cAMP-PKA signaling activity in response to nutrients and suggest that intracellular cAMP levels and dynamics can be used as a distinct molecular phenotype that captures differences not only in signaling interactions but also at genetic level.

In this work I show that in a derepressed population of *Saccharomyces* cells intracellular cAMP levels oscillate to a steady-state after an initial transient increase upon glucose replenishment. These short-term oscillations are in agreement with mathematical modeling efforts on negative feedback control of the cAMP concentrations that have also indicated decaying oscillations [138]. The level of PKA activity could be critical for this behavior because activation of PKA by increased cAMP levels is pivotal for stimulation of Ira and Pde proteins that have been implicated in initiating and mediating cAMP oscillations [109,138,144,145]. On the other hand, since decaying oscillations could be an artifact of population level examination of the cAMP

concentrations measurements at single-cell level could provide a better insight on short-term dynamics of cAMP levels.

Different oscillatory patterns of cAMP for *S. cerevisiae* wild-type and the Tpk mutant strains hint at differential interactions of PKA. The surprisingly distinct effects of TPK3 deletion on cAMP oscillations in two separate strains suggest variations in PKA may be linked to distinct oscillations. Moreover, beside considerable within species sequence variation in elements of the feedback loop between species variation is also present. Especially, the absence of the conserved cAMP phosphodiesterase class-II signature (HxHLDH[LIVM]x[GS][LIVMA][LIVM](2)xS[AP]) important for the catalytic activity of the low affinity Pde (Pde1) in *S. bayanus* is it is worth mentioning (Figure 40).

Periodicity in cAMP levels has been observed across diverse cell types and organisms at different amplitudes and frequencies. This has been proposed to be a regulatory mechanism for PKA activity in integration and transduction of diverse stimuli, and critical for coregulation of various molecular processes [139,143,146,147]. For example, highly coordinated oscillations in levels of cAMP, PKA activity and Ca^{2+} dynamics in pancreatic β-cells have been presented as a paradigm for how PKA achieves its high versatility in signal transduction [142].

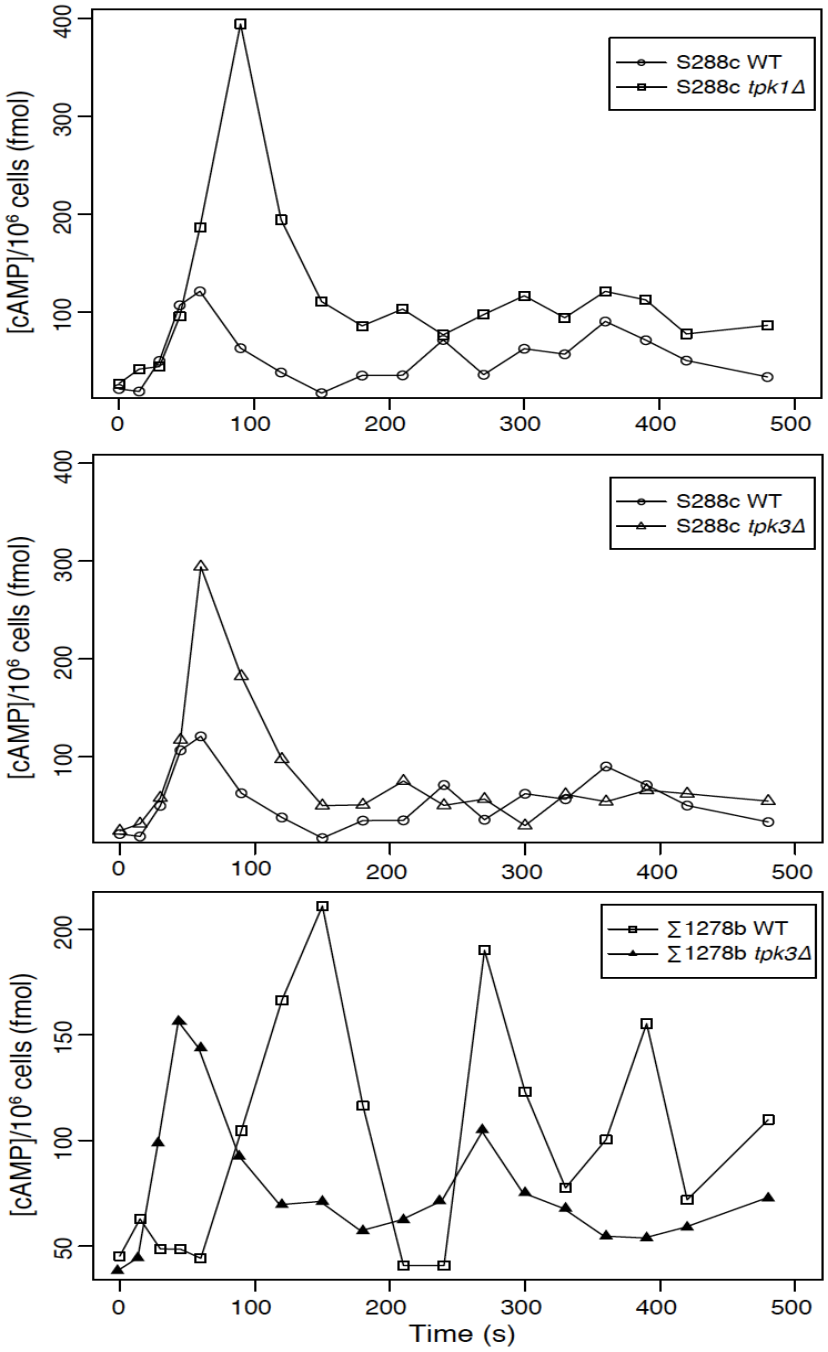

Figure 39 Oscillations capture variation in nucleotide sequence
Σ1278b has a non-synonymous SNP at catalytic domain of Tpk3

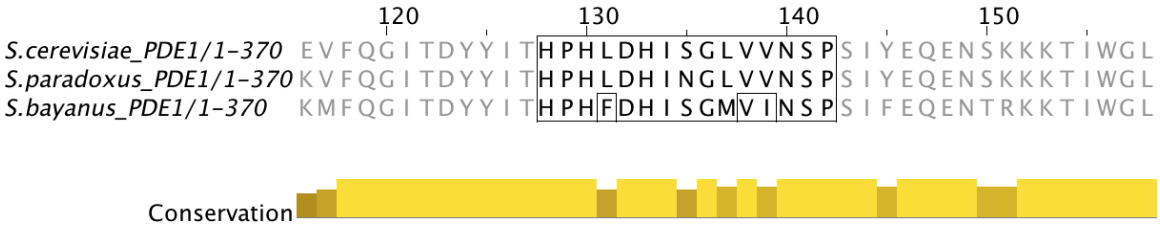

Figure 40 cAMP binding-site divergence in Pde1
The amino acid alignment for the low affinity Pde (Pde1) indicates absence of the cAMP phosphodiesterase class-II signature in *S. bayanus*.

Additionally, in yeast, although direct evidence has yet to be shown, nucleocytoplasmic oscillations of Msn2, a general stress response factor that is directly targeted by PKA in response to stress conditions, have been ascribed to oscillations in cAMP levels. Furthermore, qualitatively different oscillatory behavior of Msn2 to glucose limitation has been suggested to be due to variation in upstream signaling interactions [139,146]. Hence, although remains to be tested vigorously, observed within and between species variation in short-term cAMP oscillations may reflect variation in levels of PKA activity and interactions important to quickly adapt to nutritional changes to maximize growth and survival in their natural environments.

5. Concluding Remarks

This comparative study on the functional evolution of the cAMP-PKA signaling in closely related *Saccharomyces* species contributes to our understanding of how integration and coordination of molecular interactions govern decision-making processes both in growth and stress conditions. Conservation of the cAMP-PKA signaling in diverse organisms spanning billions of years of evolution emphasizes its importance. In this study, I show that cAMP-PKA signaling contributes to a life-history trade-off in yeast. The variation in intracellular cAMP concentrations correlates with variation in pseudohyphal growth and sporulation. These responses are alternative developmental programs to nitrogen limitation, and exhibit a phenotypic trade-off. Moreover, segregation of sporulation QTLs and expression levels for elements in the cAMP-PKA pathway are also correlated with variation in pseudohyphal growth and sporulation.

Surprisingly, despite conservation of these developmental responses in *Saccharomyces sensu stricto*, however, their regulation by cAMP-PKA signaling has clearly diverged. *S. cerevisiae* and *S. bayanus* exhibit different intracellular cAMP levels; and while pseudohyphal response is proportional to the cAMP signal in *S. cerevisiae*, it is antagonistically correlated in *S. bayanus*. Also, interestingly, Flo11 is dispensable for expression of the pseudohyphal phenotype in *S. bayanus*. In *S. cerevisiae*, Flo11 is an adhesin critical for pseudohyphal differentiation in response to nutrient stress.

Furthermore, stress- response machinery seem to operate differently between the two species. Disruption of the mechanism has opposite effects on pseudohyphal growth. The switch in regulation of nutrient-induced pseudohyphal development may have happened in response to environmental or evolutionary factors. It will be particularly interesting to know what molecular interactions have been reconfigured and how these differences correspond to natural environment of the species.

The comparative analysis presented here also suggests the potential use of intracellular cAMP dynamics as molecular phenotypes. Restoring glucose availability leads to distinct oscillatory dynamics in cAMP levels. Variation in characteristics of these dynamics may be linked to variation in nutrient sensing and development. Further examinations of these oscillations are needed to understand how these initial dynamics relate to divergence in sequence, transcriptional regulation, and developmental responses.

Elucidating the evolution of functional interactions of cAMP-PKA signaling in a comparative context will contribute insights on how developmental responses are conserved or have evolved as well as a broader understanding of how cells sense and respond to changing environmental conditions.

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Biography

Ömür. Kayıkçı was born on July 29, 1978 in Antakya, Turkey, where she lived until attended college. She studied biology at The Middle East Technical University and received her Bachelors of Science degree in 2001. She obtained her Master of Science degree from The University at Buffalo, SUNY in 2005. In 2008, she began her doctoral studies at Duke University in Durham, NC with the Biology program. She joined the laboratory of Dr. Paul Magwene in the Department of Biology. She has co-authored multiple research articles and has been selected to present her work at several scientific meetings.

Publications

Gonzales K., Ö. Kayıkçı, D. G. Schaeffer and P. M. Magwene (Accepted). Modeling mutant phenotypes and oscillatory dynamics in the Saccharomyces cerevisiae cAMP-PKA pathway (BMC Systems Biology)

Granek J. A., D. M. Murray, Ö. Kayıkçı, and P. M. Magwene (2013). The genetic architecture of biofilm formation in a clinical isolate of Saccharomyces cerevisiae (Genetics)

Magwene P. M., Ö. Kayıkçı, J. Reininga, J. Granek and D. Murray (2011). Outcrossing, mitotic recombination, and life-history tradeoffs shape genome evolution in Saccharomyces cerevisiae. PNAS 108, 1987-1992

Granek J. A., Ö. Kayıkçı and P. M. Magwene (2011).Pleiotropic signaling pathways orchestrate yeast development. Current Opinion in Microbiology 14, 676–681

Selected conference presentations

13th International Conference on Yeasts	2012
25th International Conference on Yeast Genetics and Molecular Biology	2011

Selected awards and fellowships

Summer Research Fellowship, The Graduate School, Duke University	2013
Chairman's Travel Award Department of Molecular Genetics and Microbiology, Duke University	2012

Memberships

The Genetics Society of America	2013